UNIVERSITY OF ALBERTA

EXPRESSION STUDIES OF *fem-2* GENE PRODUCTS

By

©

PETRA JÄCKLE-BALDWIN

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FACULTY OF GRADUATE STUDIES AND RESEARCH

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Abstract

Activity of the *Caenorhabditis elegans* gene *fem-2* is required for male somatic development and for spermatogenesis in males and hermaphrodites. We have studied the expression of *fem-2* gene products. Transcript expression was investigated using green-fluorescent protein (GFP) under the control of the *fem-2* promoter. The strongest GFP expression was observed in the intestine of hermaphrodites and in the somatic gonad including the distal tip cells in males. Protein expression was studied using anti-FEM-2 antiserum made for this study. Western blots showed a band of the size predicted for FEM-2 in wildtype worm extracts. This band was absent in animals homozygous for the null alleles of *fem-2*. Indirect immunofluorescence demonstrated that putative FEM-2 is most abundant in the cytoplasm of oocytes in hermaphrodites, consistent with maternally contributed *fem-2* product. Worms carrying temperature sensitive alleles of *fem-2*, raised at the restrictive temperature, showed alternative localization of FEM-2 to the nuclei of the oocytes.

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Chapter 1 Introduction

1.1 General Background

Sex determination in *Caenorhabditis elegans* is a suitable model system to investigate a question central to developmental biology: How do cells in the developing organism choose among a variety of possible differentiated fates? Specifically we are looking at *fem-2*, one gene in the sex determination pathway of *C. elegans*, and its role in causing cells to choose a male or female fate. To understand the role of *fem-2* we need to determine when and where the gene is expressed. The work presented in this thesis takes two approaches to this question. First, I examined the temporal and spatial expression pattern of a reporter gene under the control of the *fem-2* promoter. Second, I investigated when and where the FEM-2 protein product is expressed.

We have chosen *Caenorhabditis elegans* as an experimental organism for our studies of the sex determination pathway due to a number of outstanding properties it offers. The worm has a life cycle of only three days in which it progresses from the egg through four larval stages (L1- L4) to the adult (Wood *et al.* 1980). It remains fertile for approximately four days and has a total life span of about 18 days. This short life cycle together with its small size allows fast and cheap cultivation and maintenance of worm stocks on agar plates containing *E. coli* as food. For long term storage nematode stocks can be frozen in liquid nitrogen and subsequently revived (Wood, 1988).

Another advantage of *C. elegans* is its anatomical simplicity. Its body plan is typical for a nematode, consisting of an outer tube and two inner tubes. The outer tube is made up of the cuticle, the hypodermis, neurons and body wall muscles. This tube surrounds a pseudocoelomic space embodying the intestine and gonad (White, 1988). Since *C. elegans* is completely transparent we can study living worms under the light microscope using Differential Interference Contrast or Nomarski optics. Using this technique Sulston and colleagues deciphered the complete *C. elegans* lineage (Sulston *et al.*, 1983; Sulston and Horvitz, 1977). In his work Sulston showed that embryonic as well as postembryonic cell divisions follow precise and almost invariant temporal and spatial patterns, giving rise to a fixed number of cells with a determined fate. The complete cell lineage map now provides a powerful basis for any further analysis. Despite its simple organization, *C. elegans* shows the full range of differentiated cell types found in more complicated animals. The powerful resource of this extensive cell mapping allow us to determine the exact locations, cell type and times during which *fem-2* gene products are expressed.

C. elegans also offers a relatively small genome size of $1x10^8$ nucleotide pairs (Hodgkin *et al.*, 1995). Approximately 80% of the genome consists of single copy sequences, the majority of the rest is made up of moderately repetitive sequences present in two to ten copies (Sulston and Brenner, 1974). A large proportion of the moderately repetitive sequences are transposable elements such as the Tc1 element (Wood, 1988). Two commonly used strains of *C. elegans* carry vastly different numbers of these transposable elements - the Bergerac strain (BO) carries about 300 Tc1 copies while the Bristol (N2) strain carries only about 30.

The genome is divided among six chromosomes, five autosomes and one X chromosome. All *C. elegans* chromosomes are holocentric meaning that the kinetochores are distributed along the entire length of the chromosome. The total number of genes for the organism has originally been estimated to be less than 5000, but recent estimates set the number to more than 13000 genes (Brenner, 1974; Hodgkin *et al.*, 1995). The complete physical map has been assembled by overlapping cosmid and YAC clones (Coulson *et al.*, 1986; Coulson *et al.*, 1991). This provides a useful tool for the mapping of any newly discovered genes and provided an excellent starting point for the mapping of *fem-2* (Pilgrim, 1993). The sequencing of the 100000 kbp genome is predicted to be completed by the end of 1998 (Roberts, 1990; Hodgkin *et al.*, 1995).

In addition to the advantages mentioned above, *C. elegans* is also the only organism for which the complete wiring diagram of the nervous system, including all the neurons and connections, has been determined (White *et al.*, 1986). This facilitates the identification of any neurons which might express the *fem-2* transcript or protein product.

Lastly, *C. elegans* has two sexes -- male and hermaphrodite. Hermaphrodites allow self-fertilization, while males allow cross fertilization. The existence of both kinds of fertilization make genetic manipulation easier. The presence of two sexes also allows to ask a question fundamental to developmental biology: Why does an organism develop as male or female, and what determines its sex? The sexual specialization might be the most far reaching determinative decision during development of *C. elegans,* affecting 30 to 40% of the approximately 1000 cells making up the worm (Hodgkin, 1988). The question of sex determination and the answers found might be widely applicable, since it is a decision common to all groups of animals.

1.2 Sex Determination in *C. elegans*

1.2.1 Sexual Dimorphism

C. elegans has two naturally occurring sexes, males and self-fertilizing hermaphrodites (figure 1-1). Both sexes are diploid with five pairs of autosomes, but males possess only one X chromosome (X0) while hermaphrodites have two (XX). Self-fertilizing hermaphrodites can only produce meiotic products containing an X chromosome, and thus produce a brood which consists almost exclusively of self-fertilizing hermaphrodites. Males arise by two mechanisms. Firstly by X chromosome nondisjunction during gametogenesis in hermaphrodites, which occurs at a rate of approximately one in one thousand. Thus about 0.2% of the self progeny of hermaphrodites are X0 males (Hodgkin, 1985). The second way to generate males is by cross fertilization between hermaphrodites and males. Since the male sperm has a competitive advantage over the hermaphrodite sperm, broods of outcrossed hermaphrodites consist of 50% males and 50% hermaphrodites (Ward and Carrel, 1979).

Males and hermaphrodites differ greatly in size as well as in adult anatomy. Approximately 30% of the 959 somatic nuclei in hermaphrodites and 40% of the 1031 somatic nuclei in males are sexually specialized. Only 650 nuclei in the adult worm are sexually indifferent. These vast differences are manifested in obvious anatomical differences. Even though almost all tissues of the worm are affected, including the germline, the somatic gonad, the intestine, the muscles, the hypodermis and the nervous system, the changes can collectively be summarized as gearing the two different sexes towards the two different behaviors they display -- egg laying in hermaphrodites and copulation in males.

The germline and somatic gonad are descendants of a four cell gonadal primodium present at hatching. This primodium consists of the Z1 and Z4 cells, which will go on to form the somatic gonad, and the Z2 and Z3 cells, which will give rise to the germline (Kimble and Hirsh, 1979). The somatic gonad follows an invariant pattern of cell lineage, while the germline is variable. Figure 1-2 gives an overview of the gonad development in hermaphrodites and males. The descendants of Z1 and Z4 will give rise to 143 nuclei, which form the five structures of the somatic gonad in hermaphrodite: the sheath, which encapsulates the germline, the spermathecae, which stores sperm, the central uterus, junctions between the uterus and spermathecae, and lastly the anchor cell, which will control vulval development (Kimble and Hirsh, 1979). Gonadal elongation is directed by the distal tip cells (DTC) (Kimble and White, 1981). In males Z1 and Z4 will give rise to 56 nuclei, which form the three structures of the male somatic gonad: the semical vesicle, which stores mature sperm, the vas deferens, which provides a passage of sperm to the exterior, and the linker cell, which directs gonadal elongation and connects the proximal end of the gonad to the cloaca.

The germline of males and hermaphrodites is derived from the Z2 and Z3 embryonic cells and form the largest tissue by number of nuclei in *C. elegans* (Kimble and Hirsh, 1979). In both sexes germ cell proliferation continues in the distal arm of the gonad. The DTCs have been found to play a crucial role not only in gonadal guidance in hermaphrodites, but also in the control of germ cell development in both sexes (Kimble and White, 1981). Laser ablation studies have shown that the DTCs are required for continued proliferation of germ cells, for establishing the axial polarity of the germ line tissue, so that the least mature cells are at the distal end while the most mature germ cells are at the proximal end of the gonad, and for the local inhibition of germ cells from entry into meiosis. Mitosis in germ cells within the influence of the DTCs is maintained by a ligand, LAG-2, which is given off by the DTCs. This ligand binds to the germline specific receptor GLP-1 (Henderson *et al.*, 1994). Mutations in *lag-2, glp-1* or ablations of the DTCs results in a reduction of the number of germ cells and in the production of more sperm cells compared to oocytes (Kimble and Ward, 1988).

While male germ cells develop only as sperm, hermaphrodites first produce about 320 sperm cells in the fourth larval stage, before switching permanently to oogenesis, which continues throughout adulthood. In addition germ cells in males enter meiosis slightly earlier than germ cells in hermaphrodites. Overall the male germline is more prolific than the hermaphrodite germline, providing more than 2500 sperm (Hodgkin, 1983b).

The intestine of both sexes is anatomically the same, however they differ physiologically. The adult hermaphrodite intestine is specialized for yolk protein production (Kimble and Sharrock, 1983; Sharrock, 1983). Yolk proteins are secreted from all 20 intestinal cells into the pseudocoelom from where they are taken up by the gonad and incorporated into the developing oocytes. Using laser ablation studies it was found that the synthesis of yolk protein is not dependent on the presence of a gonad (Kimble and Sharrock, 1983).

All sex specific muscles are derived from the postembryonic blast cell, M (Sulston and Horvitz, 1977). In males this cell gives rise to cells which form the muscles in the tail used for copulation, in hermaphrodites it gives rise to vulval and uterine muscle cells required for egg laying.

A large difference between the sexes is seen in the neuronal and hypodermal development. Males and hermaphrodites share 294 neurons. However, since the overall wiring diagram of males is significantly more complicated than that of hermaphrodites, it has been suggested that the wiring of the common neurons is different in the two sexes (Sulston *et al.*, 1980). In addition 87 of the 381 neurons in males are sex specific (White *et al.*, 1986). Most of the sex specialized neurons in males are concentrated in the tail and are necessary for copulation. Other sex specific neurons are found in the head region, such as the cephalic companion cells (CEM). The CEM cells might be used in a special chemotaxis of males towards hermaphrodites. The 12 sex specific neurons in hermaphrodites are all necessary for egg laying.

The main differences affecting the hypodermis concern vulva formation midventrally in hermaphrodites and structures needed for copulation in the male tail. Differences in the hypodermis between the two sexes might also cause the overall size difference observed. Hermaphrodites are on average 40% longer and fatter than males.

The existence of hermaphrodites in *C. elegans* is a curious phenomena. Most related species, such as *C. remanei*, only have males and females as their two sexes. It has therefore been speculated that the *C. elegans* hermaphrodites evolved from a female ancestor (Hodgkin, 1985; Hodgkin, 1992a). Hermaphrodites might be viewed as a modified females, which make sperm for a short period of time during larval development before switching to oogenesis in adulthood. This notion of hermaphrodites as modified females has been supported by genetic analysis, since mutations in one of several genes convert hermaphrodites into females (Hodgkin, 1985).

In summary we can note that a large number of sexual dimorphisms exist between the two sexes, which affect almost every tissue in the worm. A complicated regulation is thus needed to assure the proper development of all structures in the organism. Which genes are involved in the regulation of the sex determination pathway and where is its starting point?

1.2.2 X/A Ratio

While the genetic regulation of sex determination unveils itself as very complicated, the initial signal in the sex determination pathway of *C. elegans* is clear -- the ratio of sex-chromosomes (X) to autosomes (A) (Nigon, 1951). Working with triploids and tetraploids, Madl and Herman discovered that it is truly the X/A ratio, not the absolute number of X chromosomes, that determines the sex. X/A ratios of less than 0.67 (AAA/XX) resulted in male development, while X/A ratios of more than 0.75 caused hermaphrodite development (Madl and Herman, 1979). Furthermore it was shown that partial duplications of the X chromosome could shift the male phenotype previously seen for AAA/XX animals to a hermaphrodite phenotype. This suggests that no single site on the X chromosome determines the X contribution to the X/A ratio but rather that a number of different sites along the X chromosome contribute additively (Madl and Herman, 1979; Akerib and Meyer, 1994).

So far no extensive research has been conducted on the distribution of autosomal sites contributing to the X/A ratio. It is also unknown how the X/A ratio is computed on a molecular level. Various titration models have been suggested in which the sites on the X chromosome titer away an autosomal product that is present in limiting quantities (Hodgkin, 1988). The X/A ratio therefore provides a quantitative signal to set the state of a small number of sex determination genes, which in turn are the key control in determining the sexual phenotype. Mutations in the major sex determination genes can completely transform the sexual phenotype of the worm regardless of the X/A ratio.

1.2.3 The Major Sex-Determination Genes

There are seven major sex determination genes, all of which are located on autosomes (Hodgkin and Brenner, 1977; Kimble *et al.*, 1984; Nelson *et al.*, 1978, Hodgkin, 1987b). These genes are exclusively involved in sex determination. Table 1-1 give a summary of the seven major sex determination genes. In addition there are four genes that are involved in sex determination as well as dosage compensation and a growing number of genes that play a minor role in fine tuning the sex determination pathway and its interactions with downstream pathways. I will first describe the seven major sex determination genes and then briefly elute to their interactions with the master control, dosage compensation and fine tuning genes.

The seven major sex determination genes include *transformer-1, -2, -3* (*tra*), *feminization-1, -2, -3* (*fem*) and *hermaphroditization-1* (*her*) (Hodgkin and Brenner, 1977; Kimble *et al.*, 1984; Nelson *et al.*, 1978; Hodgkin *et al.*, 1989). They can be categorized into three classes. The *tra* genes, which make up the first class, are required in hermaphrodites but not in males. Loss of function mutations in these genes result in masculinization of XX animals, but have no effect on XO animals, indicating that the wildtype function of the *tra* genes has a feminizing effect. The second class consists of the *fem* genes, which are required in males and hermaphrodites. Loss of function mutations result in feminization of XX and XO animals. The *fem* genes are needed for male soma development as well as for spermatogenesis in hermaphrodites and males. The third class, which only contains the *her-1* gene, is necessary for male somatic development. Loss of function mutations result in hermaphrodite development of XO animals.

Table 1-1. Summary of genes that influence only sex determination (modified from Kuwabara and Kimble, 1992). The following abbreviations are used: loss of function (lf), gain of function (gf) and mixed character (mc) (references are given in the body of the text).

Gene	Sex specified	Mutant phenotype	Molecular identity
her-1	Male	lf: XO animals develop as hermaphrodites gf: XX animals are incompletely masculinized	Extracellular ligand
tra-2	Hermaphrodite	lf: XX animals develop as pseudomales gf: feminization of XX animals and the germline of XO animals mc: XX animals are feminized	Transmembrane receptor
tra-3	Hermaphrodite	lf: XX animals are masculinized	Cytosolic protease substrate unknown
fem-1	Male	lf: XX and XO animals are feminized	cdc10/SW16 repeats
fem-2	Male	lf: XX and XO animals are feminized	PP2C phosphatase substrate unknown
fem-3	Male	lf: XX and XO animals are feminized gf: germline of XX animals is masculinized, no effect on soma	Novel protein binds to TRA-2 and FEM-2
tra-1	Hermaphrodite	lf: XX animals develop as males gf: XO animals develop as hermaphrodites	Zinc finger Protein Transcription factor

Before discussing details of the phenotype of the major sex determination genes, their cloning, sequencing, gene products and possible interactions, it will be helpful to take a closer look at the genetic interactions between the different sex determination mutants.

1.2.4 Epistatic Interactions

Using double and triple mutants the genetic epistatic interactions between the major sex determination genes could be determined (Hodgkin and Brenner, 1977; Nelson *et al.*, 1978; Hodgkin, 1980; Hodgkin, 1986; Doniach and Hodgkin, 1984). In summary the seven genes form a negatively regulated cascade. A model of the interactions can be seen in figure 1-3.

her-1 appears to be the first gene in the cascade which is only involved in sex determination and thus is the sex determination gene which responds most directly to the X/A ratio. It in turns negatively regulates *tra-2* and *tra-3*. *tra-2* and *tra-3* negatively regulate the *fem* genes, which brings us to a branchpoint in the sex determination pathway. In somatic tissue the *fem* genes in turn negatively regulate *tra-1*. In the germline, however, *fem* mutations are epistatic to *tra-1* mutations. Thus a *tra-1/fem-1* double mutant has a male body and gonad, but only produces oocytes (Hodgkin, 1992b).

Using this model we can predict the activity states of the major sex determination genes in XX and XO animals. XO animals have a low X/A ratio, which causes *her-1* to be active. High activity of *her-1* represses *tra-2* and *tra-3*, which allows the *fem* genes to be active. The *fem* genes in turn repress *tra-1*. The overall effect is a male soma and germline. In XX animals the sex determination control is more complex, since it requires different regulation in the soma and the germline to allow a brief period of spermatogenesis. XX animals have a high X/A ratio, which represses *her-1* and in turn allows *tra-2* and *tra-3* to have high activity levels. In the soma this represses the *fem* genes, which subsequently allows high *tra-1* activity levels resulting in a female soma. In the germline *tra-2* is initially repressed by a germline specific control

(Doniach, 1986). This allows the *fem* genes to be briefly active, thus allowing a short period of spermatogenesis. Later in development *tra-2* becomes fully active, resulting in the repression of the *fem* genes, high *tra-1* activity and the continuous production of oocytes. Taken together the regulation in XX animals results in a hermaphroditic germline in a female soma.

The model of epistatic interactions allows us to make predictions about the phenotype of different mutants, however it does not yield any information about the molecular nature of the regulatory interactions. What is known about the sex determination genes on a molecular level?

1.2.5 *her-1*

her-1 is the first gene downstream of the X/A signal to have a role solely in sex determination. The gene product of *her-1* is needed for male development (Hodgkin, 1980). Its role is probably accomplished by negatively regulating *tra-2* function (Hodgkin, 1980). Loss of function mutations in the gene result in a transformation of XO animals into hermaphrodites, while gain of function mutations result in an incomplete transformation of XX animals into males (Trent *et al.*, 1988).

At this point a brief note regarding the *C. elegans* nomenclature will help further understanding. In *C. elegans* genes are italicized, e.g. *her-1*, while the corresponding protein product carries the same name, but is capitalized, e.g. HER-1.

her-1 has been cloned and sequenced (Trent *et al.*, 1991; Perry *et al.*, 1993). Two different transcripts have been found for the gene. The transcripts seem to be sex specific, being present in XO but absent in XX animals (Trent *et al.*, 1991). The *her-1* sequence predicts a novel cysteine rich polypeptide with an amino-terminal secretion signal sequence. This suggests that HER-1 acts as an extracellular ligand. This is further supported by mosaic analysis, which revealed that the gene product of *her-1* acts cell-non-autonomously (Hunter and Wood, 1992). Together these findings strongly support the notion that

HER-1 acts as an extracellular signaling peptide, being involved in intercell signal transduction. *her-1* appears to be expressed in a large number, if not all, cells of the worm.

1.2.6 *tra-2* and *tra-3*

tra-2 and *tra-3* are needed in hermaphrodites to specify female development (Hodgkin and Brenner, 1977). Their function is presumably accomplished by suppressing the activity of the *fem* genes.

Both genes have been cloned and sequenced. Relatively little is known about *tra-3*. Its sequence predicts a protein which resembles calpain, a calcium regulated cytosolic protease, which has been found in many different species (T. Barnes, personal communication). It is unknown on which protein TRA-3 would act as a protease, however genetic evidence suggests it might cleave TRA-2 in order to activate it.

Much more is known about *tra-2*. The *tra-2* gene has been cloned and its sequence predicts a protein with the characteristics of a trans-membrane receptor (Kuwabara *et al.*, 1992). Detailed mutant analysis allows predictions about different regions of the protein. Three different classes of mutations have been identified for *tra-2* (Klass *et al.*, 1976; Hodgkin and Brenner, 1977; Hodgkin, 1980). Loss of function mutations in *tra-2* transform XX animals into non-mating pseudomales, but have no effect on XO animals (Klass *et al.*, 1976). Alleles belonging to this class of mutation have been mapped to the 5' region of the gene, a region which corresponds to the cytosolic part of TRA-2. Since loss of function mutations result in a lack of suppression of the *fem* genes, it can be presumed that the cytoplasmic region is interacting with the products of the *fem* genes. Direct evidence of protein-protein interaction have been found between TRA-2 and FEM-3 (P. Kuwabara and A. Spence, personal communication). *tra-2* gain of function mutations result in the feminization of XX animals as well as of the XO germline and intestine (Goodwin *et al.*, 1993). Gain of function mutations map to direct repeat elements of the 3' untranslated terminal region (UTR) of *tra-2* (Goodwin *et al.*, 1993). It can therefore be concluded that the sequences of the 3' UTR are used to repress *tra-2* at the translational level. This regulation might involve the *laf-1*(*l*ethal *a*nd *f*eminization of germline) gene product (J. Kimble, personal communication). It should be noted that 3' UTRs with a regulatory function have also been found for *fem-3*, however the repeat sequences are different and thus almost certainly serve a different regulatory function. The third group of mutations in *tra-2* behave like gain of function mutations, however they only affect XX animals and are thus called mixed character mutations (Goodwin *et al.*, 1993). Mixed character mutations, which map to the carboxyl terminus of TRA-2, might identify the region used in the transient inactivation of *tra-2* in hermaphrodites to allow sperm production in L4 (Doniach, 1986). In wildtype worms this inactivation might be achieved by the protein product of *fog-2* (*f*eminization *o*f the *g*ermline) and might be restricted to the germline (J. Kimble, personal communication). In addition this region might play a role in the repression of the *fem* genes.

Three different *tra-2* transcripts have been discovered, of which one might be germline specific (Okkema and Kimble, 1991). Northern blot analysis revealed much higher levels of all three transcripts in hermaphrodites than in males. Since the sex specificity of the *tra-2* mRNA always corresponds to the phenotypic sex, rather than the chromosomal sex, a feedback loop within the sex determination pathway has been suggested (Okkema and Kimble, 1991).

1.2.7 *fem-1*

fem-1, like the other *fem* genes, is required for all aspects of male development. Loss of function mutations of *fem-1* transform XX and XO animals into females (Doniach and Hodgkin, 1984; Hodgkin, 1986).

fem-1 has been cloned and sequenced (Spence *et al.*, 1990). Males and hermaphrodites showed a single transcript of 2.4 kb, which is predicted to encode a soluble, intracellular protein. Near its amino terminus six copies of the cdc10/SW16 motif were found, a motif that has been seen in a number of regulatory proteins (eg. proteins involved in cell cycle regulation) in a wide variety of organisms (Spence *et al.*, 1990). These motives are the first indication that FEM-1 is involved in protein-protein interactions.

1.2.8 *fem-2*

fem-2 is also required in males and hermaphrodites to specify male development. Together with *fem-1* and *fem-3* it is needed for male somatic development and for spermatogenesis in males and hermaphrodites (Kimble *et al.*, 1984; Hodgkin, 1986).

Twelve loss of function mutations, which result in feminization of XX and XO animals, have been isolated (Kimble *et al.*, 1984; Hodgkin, 1986). *fem-2* was cloned and sequenced during the work described in this thesis, and sequence analysis of all existing alleles could only distinguish four different mutations, suggesting a possible mischaracterization in the initial isolation (Pilgrim *et al.*, 1995). The four existing alleles include two temperature sensitive mutations, *fem-2(b245, ts)* and *fem-2(q117, ts),* and two putative null mutations, *fem-2(e2105)* and *fem-2(e2102)* (Hodgkin, 1986; Pilgrim *et al.*, 1995). The recent recharacterization of *fem-2(e2105)* as a null mutations was based on the finding that hemizygous XO animals carrying the *fem-2(e2105)* allele *in trans* to the deficiencies *Df124* or *wcDf1,* both of which delete the *fem-2* gene, were phenotypically indistinguishable from *fem-2(e2105)* homozygotes at 20°C and 15°C (Pilgrim *et al.*, 1995). Since one of the tests for a null allele is that homozygous and hemizygous animals show the same phenotype, the evidence suggests that *fem-2(e2105)* is a null allele. Earlier findings had suggested that the *e2105* allele may retain some FEM-2 activity, since homozygous *fem-2(e2105)* XO animals are completely feminized only at 25°C (Pilgrim *et al.*, 1995; Hodgkin, 1986). The location of the mutations for all four alleles of *fem-2* in respect to the five exons and four introns of the gene are shown in figure 1-4.

fem-2 exhibits a maternal rescue effect, suggesting that either *fem-2* mRNA or FEM-2 is contributed to the oocyte (Hodgkin, 1986). With genes exhibiting maternal rescue, such as *fem-2*, the phenotype of the *fem-2* mutant depends on the maternal genotype rather than its own. For instance *fem-2* homozygous mutant XX daughters of *fem-2* heterozygous mothers are self-fertile, indicating that they still produce sperm. Only *fem-2* homozygous mutant XX daughters of *fem-2* homozygous mothers exhibit full feminization. As well as maternal rescue effect XO animals show temperature sensitivity. Only homozygous *fem-2* mutant XO animals of homozygous *fem-2* mothers raised at 25°C show full feminization. Maternal product is sufficient to rescue the male somatic phenotype completely, but shows incomplete rescue of the germline. In addition to maternal effect, developing zygotes that carry a functional copy of *fem-2* exhibit zygotic rescue. In this case the functional zygotic copy of the gene is expressed resulting in a self-fertile phenotype despite of the absence of functional maternal product in the zygote.

Temperature shift experiments of *fem-2(b245ts)* mutants were used to determine the time span when the *fem-2* product was needed to fulfill its two functions in development. For germline development in XX animals, the temperature sensitive period was between L1 and L2. For germline development in XO animals, the temperature sensitive period started in L2 and continued into adulthood. For somatic development in XO animals, *fem-2* product was needed from the egg stage to L1 (Kimble *et al.*, 1984).

fem-2 has been mapped to the left end of Linkage Group III (Pilgrim, 1993). Using transformation rescue of the *fem-2* mutant phenotype with clones from this region, the location of the gene was narrowed down to a 15 kbp clone. As will be described briefly later in this thesis, this clone was used to screen a mixed stage *C. elegans* cDNA library (Barstead and Waterston, 1989) and a putative *fem-2* cDNA was isolated (Pilgrim *et al.*, 1995). The cDNA as well as the genomic copy of the gene were sequenced (Pilgrim *et al.*, 1995). Northern as well as cDNA analysis are consistent with a single transcript (Pilgrim *et al.*, 1995).

The *fem-2* sequence shares significant similarity with serine-threonine protein phosphatases 2C (PP2C) from systems as diverse as yeast, humans and plants (Pilgrim *et al.*, 1995). PP2C phosphatases are characterized by their dependency on Mg^{2+} or Mn^{2+} cations, their resistance to the phosphatase inhibitor okadaic acid and their lack of sequence similarity to the PP1/PP2A/PP2B phosphatases (Cohen *et al.*, 1989). In addition PP2Cs normally act as monomers. All putative FEM-2 homologues, about which enough background knowledge is known, have been implicated to act as phosphatases in different signal transduction pathways. The ABI1 protein from *Arabidopsis thaliana* appears to be a Ca^{2+} modulated phosphatase that links abscisic acid signals to phosphorylation dependent response pathways (Leung *et al.*, 1994; Meyer *et al.*, 1994). Abscisic acid is a plant hormone which regulates a wide variety of processes including seed maturation, dormancy and adaptation to environmental stresses. The PTC1 protein from *Saccharomyces cerevisiae* and the ptc1+ protein from *Saccharomyces pombe* appear to act as redundant inhibitors of a MAP kinase cascade regulating osmostasis (Maeda *et al.*, 1993; Shiozaki and Russell, 1994; Maeda *et al.*, 1994; Shiozaki and Russell, 1995). Nothing is known about the role of the human homologue (Nomura *et al.*, 1994). None of the other gene products indicated in the signal transduction pathways involving ABI1 in *Arabidopsis* and PTC1 in yeast show similarity to other genes in the sex determination pathway *of C. elegans.*

The homologs of FEM-2 can be grouped into three classes (figure 1-4b). All classes share six motifs with particularly high amino acid conservation. Class A, which includes FEM-2, ABI1 and the human homolog, is characterized by a long amino terminal domain, a short carboxy terminal domain and a lack of a decapeptide between conserved motives IV and V. The *Arabidospsis* ABI1 amino terminus encodes a EF-hand motif, which is related to previously characterized $Ca2^{+}$ binding sites (Leung *et al.*, 1994). This finding together with similar findings in other phosphatases suggests that the amino terminal domain might be involved in regulating the enzymatic activity of the class A

PP2C phosphatases. No sequence similarity between the amino terminal domains of class A proteins has been found. Class B proteins, which include PTC1 and ptc1+, have a shorter amino terminal domain, lack a carboxy terminal domain and have no decapeptide separating motif IV from V. Class C PP2C, which include the mammalian PP2C isoforms and an uncharacterized open reading frame from *C. elegans*, lack a long amino terminal domain, possess a larger carboxy terminal domain and a decapeptide between motives IV and V (Pilgrim *et al.*, 1995).

Recently it has been shown that FEM-2 has phosphatase activity *in vitro* and can rescue a ptc1 mutant of yeast when expressed from a plasmid under the control of a constitutive yeast promoter (D. Hansen and D. Pilgrim, manuscript submitted).

1.2.9 *fem-3*

fem-3 was initially isolated together with other sex determination mutants (Hodgkin, 1986). Along with *fem-1* and -2 it is required for male development. Lack of maternal and zygotic *fem-3* activity transforms XX and XO animals into females. *fem-3*, like *fem-2*, shows maternal effect (Hodgkin, 1986). In this case it is known that the maternal effect is caused by *fem-3* mRNA, which is contributed to the embryos as maternal product (Barton *et al.*, 1987). A number of *fem-3* gain of function alleles have been isolated as suppressor mutations of *fem-1* and -2 loss of function alleles. All *fem-3* gain of function alleles result only in the masculinization of the hermaphrodite germline, but have no effect on the soma. These alleles exhibit a temperature sensitive period from late L4 to early adult. Together these results suggest that in wildtype hermaphrodites *fem-3* is negatively regulated to switch hermaphrodites in late L4 from sperm to oocyte production (Barton *et al.*, 1987). The temperature shift experiments has also shown that sexual commitment in the germline is a continuing process, so that spermatogenesis can be induced by a temperature shift even after oogenesis has already started.

Three *fem-3* transcripts of different sizes have been identified. The largest transcript was embryo specific and exhibited a very long poly(A) tail (Barton *et al.*, 1987; Ahringer *et al.*, 1992). A long poly(A) tail might be required for the *fem-3* mRNA to be active. All *fem-3* gain of function alleles were found to carry mutations in a five base pair region in the 3' UTR of the gene (Ahringer and Kimble, 1991). This suggest that the *fem-3* mRNA is regulated through binding a factor to its 3' UTR that inhibits translation. This binding might result in a shortening of the poly(A) tail and thus in inactivation of *fem-3* (Ahringer and Kimble, 1991).

The highest levels of *fem-3* mRNA were observed during embryogenesis and adulthood (Rosenquist and Kimble, 1988). It was also found that *fem-3* mRNA production in adult hermaphrodites depended on the presence of the germline and thus most likely is synthesized in this tissue (Rosenquist and Kimble, 1988). Equal amounts of *fem-3* mRNA were found in XX and XO embryos, indicating that sex-specific regulation of maternal *fem-3* activity has to occur post-transcriptionally (Ahringer *et al.*, 1992). The *fem-3* ORF encodes a novel protein, which is predicted to act intracellularly.

Recently it has been found that FEM-3 interacts with TRA-2 as well as with FEM-2, confirming previous evidence that the *fem* gene products act in the cytoplasm and are probably involved in transferring a signal from the cell surface receptor TRA-2 to the nucleus (P. Kuwabara and A. Spence, personal communication).

1.2.10 *tra-1*

tra-1 has a central role in the sex determination pathway. Like the *fem* genes it is involved in two developmental decisions. In the germline *tra-1* wildtype activity promotes oogenesis and inhibits spermatogenesis (Schedl *et al.*, 1989). In the soma it promotes female development, probably by activating female specific genes and repressing male specific genes. A total of 43 loss of function and 22 gain of function mutations have been described (Hodgkin, 1987a). Strong *tra-1* gain of function mutations transform XO animals into fertile hermaphrodites, while strong *tra-1* loss of function mutations transform XX animals into fertile males. It is thus possible to construct strains which sex is determined by the state of the *tra-1* locus rather than the X/A ratio (e.g. *tra-1 (gf)/ +; tra-3/tra-3* females and *+/+; tra-3/tra-3* males) (Hodgkin, 1983a).

tra-1 has been cloned, and two transcripts of 1.5 kb and 5 kb have been isolated (Zarkower and Hodgkin, 1992; Hodgkin, 1992b; Hodgkin, 1993). The transcripts encoded two and five Zinc finger motifs of the C2H2 (cysteine, histidine) class respectively. This suggests that TRA-1 acts as a transcription factor (Zarkower and Hodgkin, 1993). Most mutations mapped so far affect only the longer transcript, indicating that it is necessary. *In vitro* binding assays have shown that the 5 kb mRNA encodes a protein which can bind to DNA. No *in vitro* binding was observed with the protein product of the 1.5 kb mRNA (Zarkower and Hodgkin, 1992). In agreement with its function as a transcription factor, it had previously been shown by mosaic analysis that *tra-1* acts cell-autonomously in the soma (Hunter and Wood, 1990).

The 5 kb transcript is present at constant levels throughout development, while the 1.5 kb transcript peaks at L2, with much reduced levels at the other stages. These observations allow several possible modes of action for the smaller mRNA. One possibility is that it acts as a transcription factor with slightly different specificity. Alternatively it might only enhance the activity of the larger mRNA, such as by titering out negative regulators (Zarkower and Hodgkin, 1992). No difference in mRNA levels were observed between the two sexes, suggesting that *tra-1* regulation occurs primarily post-transcriptionally.

Mutant analysis of *tra-1* revealed some important regulatory regions. Gain of function mutation of *tra-1* result in somatic feminization of XX and XO animals regardless of the upstream sex determination signal, indicating a lack of negative regulation of *tra-1* in male tissues (de Bono *et al.*, 1995). All the gain of function mutations fall into the same region of *tra-1* at the 5' end of the gene and are proposed to cause missplicing. This eliminates a region of the protein that carries a phosphorylation site for a glycogen synthase kinase. These findings suggest that TRA-1 is posttranslationally inhibited by phosphorylation or by protein protein interactions (de Bono *et al.*, 1995).

1.2.11 The Molecular Model

Many reasons for such a complicated cascade to determine the sex of *C. elegans* have been suggested. One widely accepted hypothesis is that the cascade can act as a correcting mechanism for cells that abberantly assess their X/A ratio. Through signals from the neighboring cells, the cell can subsequently still adapt the correct sexual fate (Hunter and Wood, 1992). Other hypotheses take into consideration that *C. elegans* has to not only specify male and female somatic development, but in addition has to allow a limited time of spermatogenesis in hermaphrodites. This added difficulty in regulation might add much to the complexity of the sex determination pathway of *C. elegans*, a complexity that would not be required in a species with only males and females (Hodgkin *et al.*, 1985).

Through the findings of many different laboratories an overall picture of how sex determination might work on a molecular level is emerging. This model is constantly changing as new findings from the sex determination pathway are presented. The most current model is shown in figure 1-5. In this model HER-1 acts as an extracellular ligand (Trent *et al.*, 1991), binding to the transmembrane receptor TRA-2 (Kuwabara *et al.*, 1992), and thereby inhibiting its interaction with the FEM proteins. How the three FEM proteins may interact is not resolved at this time, however interactions between FEM-3 and TRA-2 as well as FEM-2 have been found (P. Kuwabara and A. Spence, personal communication). This suggests that FEM-3 might bind the FEMs to TRA-2, whenever TRA-2 is not bound to HER-1, thereby inactivating them. Whenever TRA-2 binds HER-1 the FEMs are released and thus can perform their enzymatic role in the cell. It is not clear whether they act on an intermediate gene product in the cytoplasm thus relating the message from the cell surface to the transcription factor TRA-1 (Zarkower and Hodgkin, 1992), or whether they act directly on TRA-1 in the nucleus. Since no intermediate genes have been found in the sex determination pathway, it is likely that the FEMs interact directly with TRA-1. Recent findings of a protein binding domain at the 5' end of TRA-1 adjacent to a phosphorylation site support this hypothesis (de Bono *et al.*, 1995). This domain allows the possibility that FEM-1, which also has a protein-protein binding domain, might bind the active FEMs to TRA-1. Once bound FEM-2, which has been found to have phosphatase activity (Pilgrim *et al.*, 1995; D. Hansen, personal communication), might dephosphorylate TRA-1 thus inactivating it. When the FEMs are bound to TRA-2, and thus inactive, TRA-1 would be phosphorylated and could act as active transcription factor. The shortcoming of this model is that no kinase has been identified yet and that it has previously been suggested that the inactive form of TRA-1 might be phosphorylated (de Bono *et al.*, 1995). Alternatively FEM-2 could dephosphorylate FEM-1, thereby activating it. At this point it remains to be shown which protein FEM-2 acts on, and which proteins FEM-1 and TRA-1 interact with.

1.2.12 Fitting the Sex Determination Pathway into Context

In recent years a lot of evidence has accumulated that indicates that interactions between the pathway formed by the seven major sex determination genes and other pathways are very complex. Figure 1-6 fits the epistatic pathway formed by the seven major sex determination genes into context and captures a simplified version of the complex overall pathway that has emerged.

For completeness I will briefly mention the major groups of other genes interacting with the sex determination pathway. There are a number of genes that are involved in sex determination as well as dosage compensation and act upstream of the major sex determination genes. Genes involved in both pathways include *xol-1, sdc-1, -2* and *-3* (Miller *et al.*, 1988; Nusbaum and

Meyer, 1989; Villeneuve and Meyer, 1990; DeLong *et al.*, 1993). *xol-1* (*XO-lethal*) is thought to most directly respond to the X/A ratio very early in embryogenesis (28 cell stage) and in response set the sexual fate of the animal by negatively regulating the *sdc* (*s*ex determination and *d*osage *c*ompensation) genes (Rhind *et al.*, 1995). Continued assessment of the X/A ratio throughout development does not seem necessary. High levels of *xol-1* transcript during gastrulation specify male development, while low levels specify hermaphrodite development. *xol-1* mutations results in embryonic or early L1 lethality in XO animals caused by the disruption in dosage compensation, but has no effect on XX animals (Miller *et al.*, 1988).

The target of the *xol-1* gene products, the *sdc* genes, form a branchpoint between the dosage compensation and the sex determination pathway. They repress the transcript levels of *her-1* and activate the XX-specific dosage compensation genes *dpy-21, -26, -27, -28* and *-30* (Kuwabara and Kimble, 1992; Hsu and Meyer, 1993). Mutations in *sdc-1* and *-2* cause masculinization and increased levels of X-linked gene expression in XX animals, the latter causing XX-specific lethality (DeLong, 1993). In *C. elegans* dosage compensation is accomplished by down regulating the level of X-linked gene expression of both X chromosomes in XX animals as compared to XO animals (Meneely, 1990). This downregulation appears to be accomplished by changing the higher order chromosome structure. *dpy-27*, one of the dosage compensation genes, for instance, encodes a chromosome condensation protein homolog (Chuang *et al.*, 1994). The genes just described act upstream or parallel to the sex determination pathway, but what is known about genes acting downstream of the pathway?

In a developmental pathway, one can regard one set of genes as the selector genes, genes which chose between different possibilities, while another set of genes can be regarded as realizator genes, genes which execute those possibilities (Garcia-Bellido, 1975; Hodgkin *et al.*, 1985). Mutations in selector genes will result in developmental transformations, while mutations in realizator genes will result in defective development without causing transformations. For our study the *fem* and *tra-1* genes act as selector genes, which will target downstream genes to execute one particular part of the development specified. Examples of realizator genes include the yolk-protein (Kimble and Sharrock, 1983) and sperm-protein genes, which are involved in the final sexual differentiation of a specific tissue. The realizator genes, however, also include genes which themselves are connected to a whole differentiation pathway. Examples of this class include the *mab-3* and *-9* genes (*m*ale-specific *ab*normality), which affect the male soma development (Hodgkin, 1983b; Shen and Hodgkin, 1988), and the *lin* genes, which affect vulva formation in hermaphrodites (Ferguson and Horvitz, 1985). Other genes in this category are involved in germline specific functions, but have no effect on the soma, such as the *fog-2* and *-3* gene (*f*eminization *o*f *g*ermline) (Schedl and Kimble, 1988; Ellis and Kimble, 1995) and *mog* genes (masculinization *o*f *g*ermline) (Graham and Kimble, 1993; Graham *et al.*, 1993).

1.3 Statement of Goals

When I started my graduate project in Dr. Pilgrim's laboratory a genomic region which rescued the *fem-2* mutant phenotype had been cloned, and cDNAs mapping to the region had been isolated (Pilgrim *et al.*, 1995). Analysis of the mutant alleles of *fem-2* as well as the spatial and temporal expression of the *fem-2* mRNA was under way (Pilgrim *et al.*, 1995). The next logical step was to determine the expression pattern of the FEM-2 protein and to compare it to the expression pattern observed in transgenic worms containing a *fem-2* - reporter gene construct.

My project had three parts. The first part was to prove that the cDNA we had isolated was indeed *fem-2* cDNA. This could be shown by further narrowing down the rescuing region of *fem-2* and by showing that a plasmid, which previously rescued the *fem-2* mutant phenotype, would fail to do so if a new mutation was introduced within the coding region of the gene.

The second part of the project was to study the expression patterns seen in worms carrying a reporter gene under the control of the *fem-2* promoter. As reporter gene I chose the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* (Prasher *et al.*, 1992), which fluoresces bright green whenever illuminated with blue light without the need of any other components added to the system. Many vectors using GFP as reporter gene have been constructed to follow *C. elegans* gene expression in living animals throughout development (M. Chalfie, personal communication; A. Fire personal communication).

The last and most important part was to make an antibody against FEM-2 and subsequently study the temporal and spatial expression of the FEM-2 protein in wildtype as well as mutant nematodes using Western blots and indirect immunofluorescence. The project was designed to give us further information when and where FEM-2 is found in the animals and within cells. It was aimed at getting us a step closer to discovering with which proteins FEM-2 interacts and how *fem-2* and its gene products fit into the sex determination pathway.

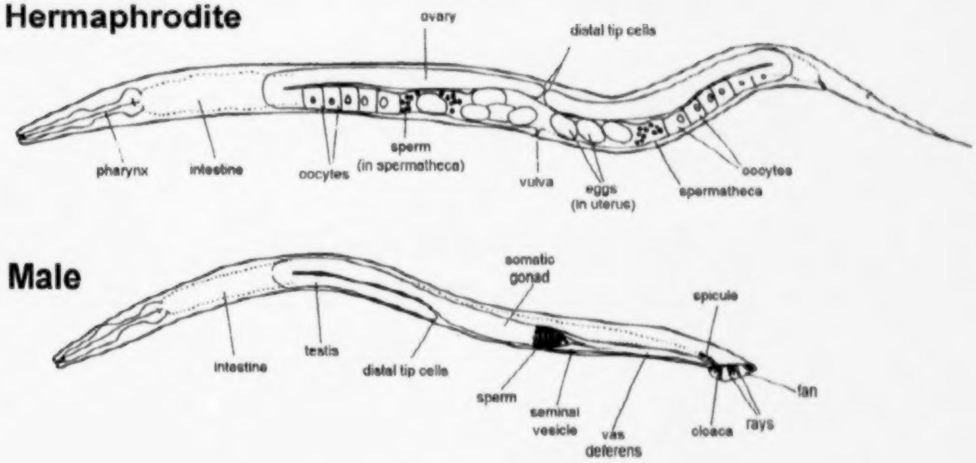

Hermaphrodite
ovary
distal tip cells
pharynx
intestine
oocytes
sperm
(in spermatheca)
vulva
eggs
(in uterus)
spermatheca
oocytes
Male
somatic
gonad
spicule
intestine
testis
distal tip cells
sperm
seminal
vesicle
vas
deferens
cloaca
rays
fan

Figure 1-1. Diagrams of hermaphrodite and male anatomy (adapted from Hodgkin, 1988).

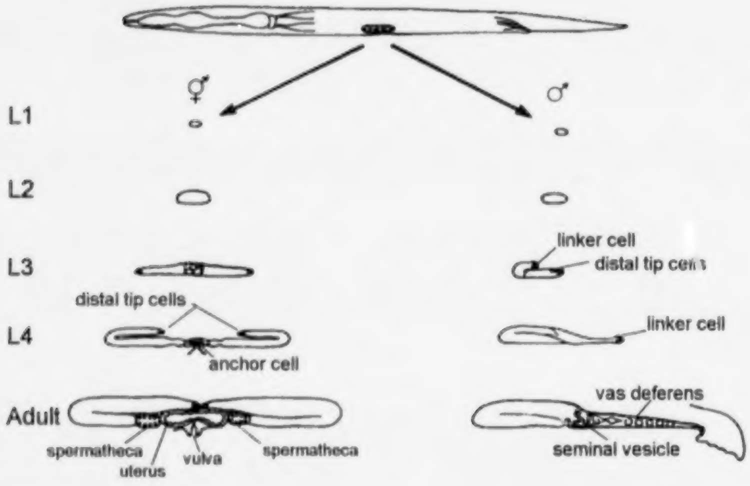

⚥
♂
L1
L2
L3
linker cell
distal tip cells
distal tip cells
L4
linker cell
anchor cell
vas deferens
Adult
spermatheca
uterus
vulva
spermatheca
seminal vesicle

Figure 1-2. Overview of gonad development in males and hermaphrodites starting with the four cell gonadal primodium present at hatching (adapted from Kimble and Hirsh, 1979). The four cell gonadal primodium consists of the cells Z1, Z2, Z3 and Z4. The descendants of Z1 and Z4 will form the somatic gonad, while the descendants of Z2 and Z3 will form the germline.

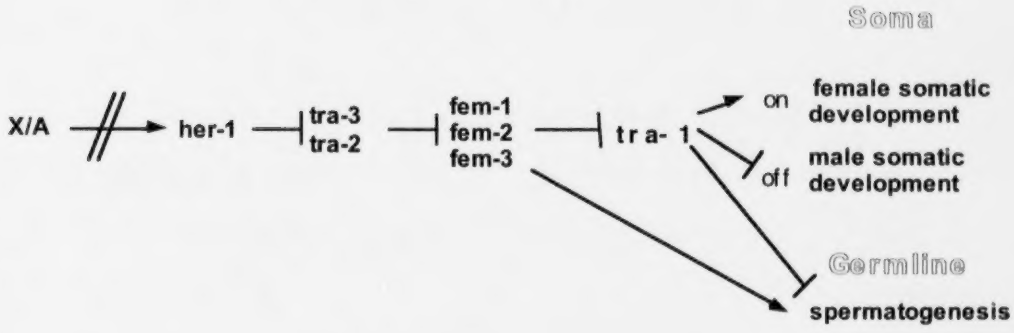
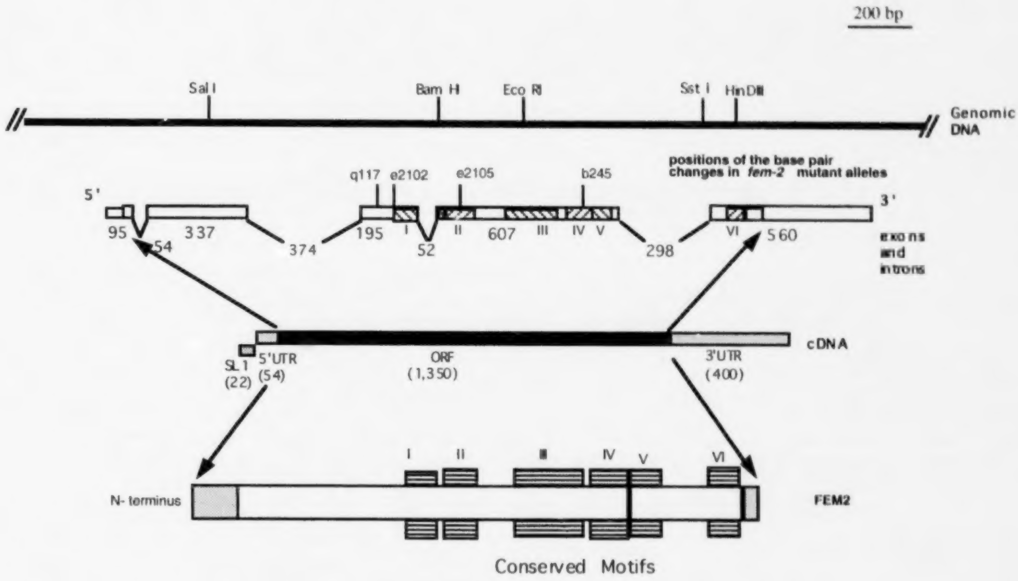

Soma
X/A
her-1
tra-3
tra-2
fem-1
fem-2
fem-3
tra-1
on
female somatic development
off
male somatic development
Germline
spermatogenesis

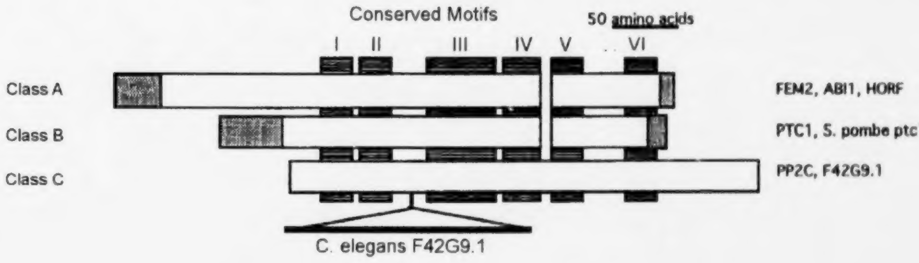
Figure 1-3. Model of genetic interactions of the *C. elegans* genes involved only in sex determination. Arrows indicate positive regulation, while bars indicate negative regulation (adapted from Hodgkin, 1992).

Figure 1-4. (a) The genomic, cDNA and protein product of *fem-2* are shown. Exons and introns as well as the location of the four known mutations of *fem-2* are indicated. Numbers refer to the number of nucleotides present in each intron, exon, ORF, UTR and SL1. UTR refers to untranslated terminal region. SL1 refers to the nematode splice leader sequence 1. The regions of conservation between PP2C phosphatases are depicted with striped boxes (adapted from Pilgrim *et al.*, 1995). (b) FEM-2 alignment with putative homologs. Conserved motifs I-VI are shown with stripped boxes (adapted from Pilgrim *et al.*, 1995). ABI1, *ABI1* protein from *Arabidopsis thaliana*; HORF, predicted human protein from cDNA sequence; PTC1, *Saccharomyces cerevisiae* product of *PTC1* gene; PP2C, rat protein phosphatase 2C β isoform; F42G9.1, ORF from *C. elegans* sequencing project (references are given in the text). Sequence accession numbers: *ABI1*, gp X77116; HORF, gp D13640; *PTC1*, sp P35182; PP2C, sp P35815 and F42G9.1, gp U00051.

a.


200 bp
Sal I
Bam H
Eco RI
Sst I
HinDIII
Genomic DNA
positions of the base pair changes in fem-2 mutant alleles
q117
e2102
e2105
b245
5'
3'
95
54
337
374
195
I
52
II
607
III
IV
V
298
VI
560
exons and introns
cDNA
SL1 (22)
5'UTR (54)
ORF (1,350)
3'UTR (400)
I
II
III
IV
V
VI
N- terminus
FEM2
Conserved Motifs

b.


Conserved Motifs
50 amino acids
I
II
III
IV
V
VI
Class A
Class B
Class C
FEM2, ABI1, HORF
PTC1, S. pombe ptc1+
PP2C, F42G9.1
C. elegans F42G9.1

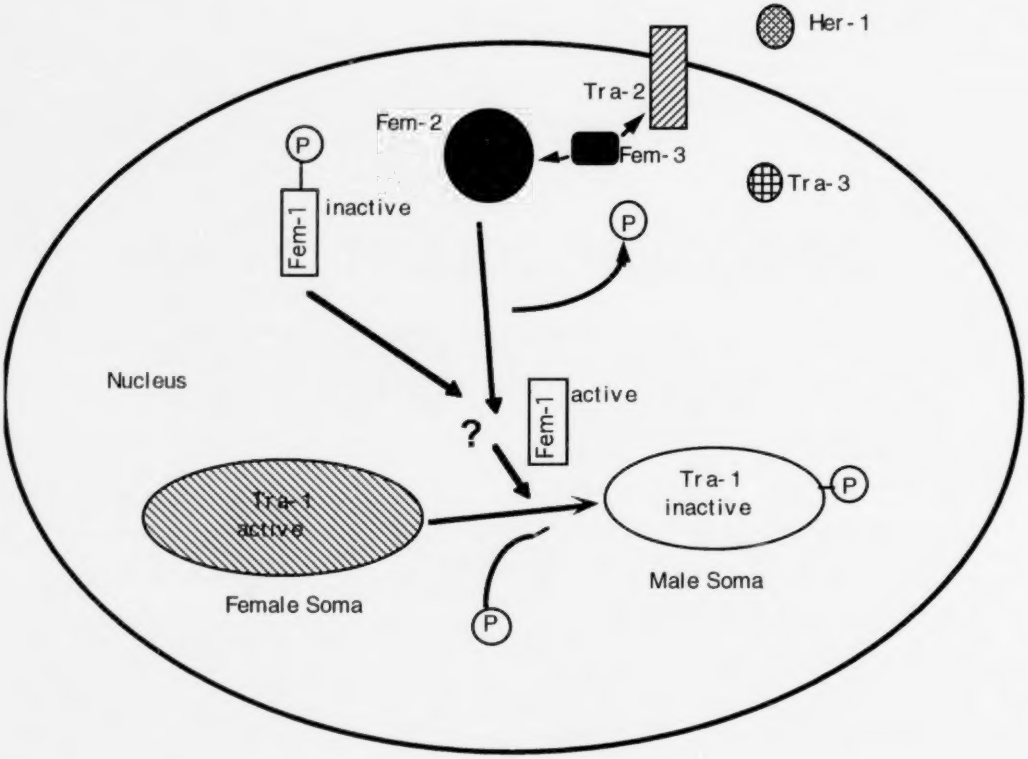

Her-1
Tra-2
Fem-2
Fem-3
Tra-3
P
Fem-1
inactive
P
Nucleus
?
Fem-1
active
Tra-1
active
Tra-1
inactive
P
Female Soma
Male Soma
P

Figure 1-5. One current molecular model of the sex determination signal transduction pathway (adapted from Kuwabara and Kimble, 1992). In the model HER-1 acts as extracellular, diffusible ligand (Perry *et al.*, 1993), TRA-2 as transmembrane receptor (Kuwabara *et al.*, 1992), the FEM genes in relating the message from the cell surface to the nucleus and TRA-1 as nuclear transcription factor (Zarkower and Hodgkin, 1992). A detailed description of the suggested interactions between the proteins is given in the text.

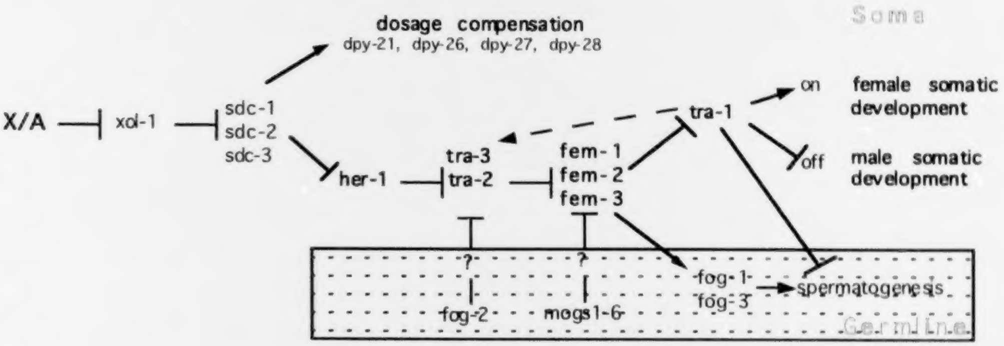

Soma
dosage compensation
dpy-21, dpy-26, dpy-27, dpy-28
X/A
xol-1
sdc-1
sdc-2
sdc-3
her-1
tra-3
tra-2
fem-1
fem-2
fem-3
tra-1
on
female somatic development
off
male somatic development
?
?
fog-2
mogs1-6
fog-1
fog-3
spermatogenesis
Germline

Figure 1-6. Regulatory pathway of genes involved in *C. elegans* germline and somatic sex determination. The seven genes highlighted are only involved in sex determination and form the core of the pathway. Arrows indicate positive regulation, while bars indicate negative regulation. Dashed lines indicate possible interactions, which are not fully confirmed. Genes within the shaded box are germline specific (modified from Kuwabara and Kimble, 1992; Hodgkin, 1992).

Chapter 2 Materials and Methods

2.1 *C. elegans* Strains and Culture Conditions

Strains used are described in table 2-1. *C. elegans* strain Bristol (N2) is the reference wild-type strain.

Table 2-1. *C. elegans* strains used in this work

Strain name	Strain genotype
CB3535	*fem-2(b245, ts)*
JK663	*fem-2(q117, ts)*
DP88	*fem-2(e2105);+/sC1(s2073);dpy-1(s2171)*
DP66	*fem-2(e2102);+/sC1(s2073);dpy-1(s2171)*
DP47	*him-8(e1489)*
BA17	*fem-1(hc17, ts)*
CB3843	*fem-3(e1996)/unc-24(e138);dpy-20(e1282)*

C. elegans stocks were maintained and handled as described by Brenner (1974) and Wood (1988). The characterization of the *fem-2* alleles used in this work was previously described by Kimble *et al.* (1984) and Hodgkin (1986). Unless otherwise indicated, the strains were maintained at 20°C. Males were obtained from a high incidence of males (*him*) strain, which produces a brood consisting of one third XO animals due to X-chromosome non-disjunction (Hodgkin *et al.*, 1979).

2.2 DNA Transformation of *C. elegans*

Transformations by microinjection of DNA were performed on young adult hermaphrodites as described by Mello *et al.* (1991). The DNA injection mixture contained the test DNA suspended in TE (10 mM Tris-Cl pH 7.4, 1 mM EDTA pH 8.0) as well as the reporter plasmid pRF4, which contains the dominant *rol-6(su1006dm)* marker (Kramer *et al.*, 1990). The *rol-6* marker causes the animals to move in a rolling fashion, an easily identifiable phenotype. The molar ratios between the test plasmid and pRF4 varied for different injections. Plasmids tested for their ability to rescue *fem-2* mutant phenotype were injected into *fem-2(b245, ts)* hermaphrodites. The worms were allowed to recover at 20°C overnight and subsequently transferred to 25°C, the restrictive temperature. The F1 progeny were examined for the rolling phenotype as well as for self fertility.

Plasmids used to monitor the expression of the *fem-2* promoter linked to the green fluorescent protein (GFP) reporter gene were injected into N2, *fem-1(hc17,ts)* and *him-8(e1489)* hermaphrodites. Rol-6 progeny from the F2 and later broods were examined for GFP expression by fluorescence microscopy as described below. To confirm presence of the correct GFP plasmid in the transgenic worms, polymerase chain reaction (PCR) was performed on the plasmids as well as on single transgenic worms (data not shown). Transformed animals will maintain the transgene extrachromosomally and will show a mosaicism of transgenic expression (Mello *et al.*, 1991). Worms with integrated transgenes arise spontaneously at low frequency or can be produced by γ-radiation (Fire, 1986).

2.3 PCR methods

Single worm PCR analysis was performed as described by Williams *et al.* (1992) and Pilgrim and Bell (1993) using the following primers: TJ01, AMC1, AMC3, MMA8, DHA3 and Reverse primer (table 2-1). All transformant lines obtained, except for one line containing a GFP plasmid, did not integrate the plasmid but maintained it extrachromosomally as judged by the meiotic instability of the Rol-6 phenotype (Mello *et al.*, 1991).

Table 2-2. Primers used in this study.

Primer	Use	Sequence (5' to 3')
TJ01	Single worm PCR	AACCACTACCGGCTTCGGTGGGACAGCTGG
AMC1	Single worm PCR	ACCTCGACGTCATAGCTAGT
AMC3	Single worm PCR	GATCACATCGGCATCGCCGTCG
MMA8	Single worm PCR	CTTTGGGTCCTTTGGCCAATCC
DHA3	Single worm PCR	CAAAGATCTTGTCCCACCGAAGCCGGTAGTGG
DHA14	Sequencing across insertion junction in pDP#PJB1	CGCGATCGACAAGATTCTCCA
H2	Sequencing across deletion in DP#DBP023ΔBamHI	GCTTCGCAAGTCGCTGGAGCTTC

2.4 DNA Manipulation

Standard procedures for DNA isolation and manipulation were followed unless otherwise indicated (Sambrook *et al.*, 1989). The sequencing was done using a Sequenase v2.0 DNA sequencing kit (United States Biochemical, Cleveland, OH).

2.5 Microscopy

To examine nematodes for GFP reporter gene or protein expression studies, worms were either anaesthetized in 10 μl of 1% 1-phenoxy-2-propanol (Koch-Light Laboratories Ltd.) directly on the glass slide or on 2% agar pads (Sulston and Hodgkin, 1988) or they were immobilized with a solution of 0.67% azide. Animals were examined on a Zeiss Axioskop microscope equipped with Differential Interference Contrast (DIC) optics and an attached halogen lamp for incident-light fluorescence. Worms examined for GFP expression or immunofluorescence were observed under the fluorescein isothiocyanate (FITC) filter. All worms that showed fluorescence were also observed under Nomarski microscopy to identify the structures which showed expression. Pictures were taken with a mounted Zeiss MC 80 microscope camera and Kodak Ektachrome slide film, ASA 400.

2.6 Construction of *fem-2* promoter-green-fluorescent-protein (GFP) vectors

Four vectors were constructed containing different fragments of the *fem-2* gene as well as different forms of GFP (Prasher *et al.*, 1992). The plasmids are shown in figure 2-1, 2-2, 2-3 and 2-4. In summary, vector pDB#PJB2 has 2.3 kbp of *fem-2* upstream region and the first three exons and introns (1.1 kbp) of the *fem-2* gene inserted into TU#62, a GFP reporter gene vector, which in turn is based on the lacZ fusion vector pPD22.04 (Fire *et al.*, 1990; Chalfie *et al.*, 1994). pDB#PJB3 is also based on TU#62 and contains the same fragment of *fem-2* upstream sequence, but only encompasses the first exon, part of the second exon, and the first intron (0.3 kbp) of the *fem-2* gene. pDB#PJB4 has the same insert as pDB#PJB3 but uses pPD95.69, a vector containing a modified GFP gene (A. Fire, Fire Lab Vector Kit, personal communication). The difference between TU#62 and pPD95.69 is the introduction of two mutations and three introns of 50 bp each in the GFP gene of pPD95.69. The modifications are reported to result in stronger fluorescence, faster folding of the fluorochrome and improved resistance to fading and therefore facilitates the detection of GFP in transgenic organisms (A. Fire, personal communication). In addition, pPD95.69 contains the nuclear localization signal of the SV40 T antigen. pDP#PJB5 is based on pBluescript with an insertion encompassing 1.3 kbp of the *fem-2* upstream region and the entire *fem-2* gene as well as the GFP gene isolated from pPD95.69. pDP#PJB5 was obtained from Dave Hansen and Morris Maduro (University of Alberta).

2.7 GST-FEM-2 Fusion Protein Production and Isolation

The Glutathione-S-transferase (GST)-FEM-2 expression vector, pDP#PJB1, was constructed by ligation of a *fem-2* cDNA from *C. elegans* (Pilgrim *et al.*, 1995) into the pGEX-1λT vector (Pharmacia LKB Biotechnology Inc.). The pGEX vector was modified prior to ligation to the cDNA by insertion of a SalI site into the EcoRI site of the polylinker using the oligonucleotide AATTCGTCGACG. The cDNA fragment used in the construction of the expression vector lacks the first 156 base pairs of the *fem-2* cDNA and starts with the SalI site at the 5' end of the gene (Figure 2-5). The orientation of the insert was confirmed using restriction digests. The reading frame of the cDNA was confirmed by sequencing across the vector/insert junction using the primer DHA14 (table 2-2).

The vector was transformed into JM105 *E. coli* cells. Fusion protein induction and isolation was performed as previously described by Rasmussen and Garen (1993). Briefly, a 400 ml culture of *E. coli* cells carrying the vector were grown at 37°C to an OD600 between 0.6 and 1. At that point fusion protein expression was induced by the addition of 1 mM isopropyl-1-thio-beta-

D-galactopyranoside (IPTG) for 3 to 5 h at 37°C. Bacteria were harvested by centrifugation and resuspended in 5 ml of lysis buffer (40 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) and 10 μg/ml each of leupeptin (Sigma), aprotinin (Sigma) and tosylphenylalanyl chloromethyl ketone (TPCK) (Sigma)). The cells were disrupted by sonication and the sonicate was isolated by centrifugation. For the isolation step the sample was mixed with an equal volume of Phosphate-Buffered Saline 0.1% Triton X-100 (PBST) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH7.3, 0.1% Triton X-100) and bound to a glutathione-Sepharose 4B column as judged by the OD280 of the column effluent. The column was washed with PBS until no more protein washed off the column. The fusion protein was eluted with 5 mM glutathione, 50 mM Tris-HCl, pH 8.0 (GST Gene Fusion System, Pharmacia Biotech). Fractions containing the GST-FEM-2 fusion protein were pooled and concentrated using Centricon-30 concentrator units (Amicon, Inc., Beverly, MA). For some experiments, GST-FEM-2 fusion protein was isolated using the batch method described in the manual "GST Gene Fusion System" (Pharmacia Biotech). In this method, the cleared sonicated lysate was added to 1.5 ml of washed, swelled GST-agarose beads. Proteins were allowed to bind to the beads at 4°C for one to two hours. The beads were pelleted, washed three times with lysis buffer (100 mM NaCl, 2.5 mM EDTA, 0.1% Tween-20, 50 mM Tris-Hcl pH 8.0, 1 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin), once with XB (0.25 sucrose, 0.1 M NaCl, 2.5 mM $MgCl_2$, 20 mM Hepes-KOH pH 7.2) and three times with washing buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0). Proteins were eluted using washing buffer containing 10 mM glutathione. Pooled elutants were dialyzed against washing buffer and subsequently against dialysis buffer B (50 mM NaCl, 20 mM Hepes-KOH pH 7.5, 20% glycerol, 10% polyethyleneglycol).

2.8 Antiserum Production Against GST-FEM-2 Fusion Protein

The crudely purified fusion protein was separated by gel electrophoresis on 8% SDS-PAGE gels (Sambrook *et al.*, 1989). To visualize the protein bands the gel was stained with 0.05% Coomassie brilliant blue R-250 (BioRad) in deionized water for ten minutes. The gel was subsequently washed for several hours in water until the band was clearly visible (Harlow and Lane, 1988). In addition a strip of the side of the gel containing a size marker lane as well as some of the sample was stained with 0.05% Coomassie brilliant blue R-250 in 40% methanol and 10% glacial acidic acid. After destaining, the strip could be compared to the remaining gel to help in the correct identification of the fusion protein band. After excising a slice of the gel containing the fusion protein band, it was homogenized by passing it through a syringe with a 21-gauge needle (Harlow and Lane, 1988). The homogenized gel slab containing about 500 mg of GST-FEM-2 fusion protein was mixed with 500 ml of Freund's complete adjuvant (Sigma) and injected intramuscularly into Flemish giant lop-eared rabbits. Injections were handled by Biosciences Animal Services of the University of Alberta. Two boost injections with 500 mg fusion protein mixed with 500 ml of Freund's incomplete adjuvant were administered 29 and 74 days after the initial injection. A "preimmune" blood sample was taken prior to the initial injection. Testbleeds were taken one week after each boost injection. A total of 40 ml of serum was harvested from each rabbit five weeks after the final injection. The blood was stored at 4°C overnight to coagulate and then centrifuged to isolate the serum. The serum was frozen in aliquots at -80°C.

2.9 Antibody Specificity Tests and Antibody Purification

Two ammonium sulfate precipitations were performed on the rabbit anti-serum using standard procedure (Harlow and Lane, 1988) to increase serum specificity to the FEM-2 protein. Briefly, saturated ammonium sulfate solution

(100%) was added to the gently stirring serum at 4^{0}C until a final concentration of 33% was reached. The mixture was stirred at 4°C overnight, then centrifuged at 3000 g for 30 minutes. The supernatant was transferred to a new beaker and saturated ammonium sulfate solution was added to a final concentration of 50%. The solution was centrifuged at 3000 g for 30 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 10 ml of PBS. The antibody solution, with sodium azide added to 0.02%, was dialyzed against three changes of PBS overnight. To further increase antibody specificity, antibody was isolated against purified full length FEM-2. GST-FEM-2 fusion protein was digested with Thrombin as described in the GST Gene Fusion System manual (Pharmacia Biotech). The following modifications were made. Fusion protein was obtained from 800 ml of bacterial culture using the batch method described above (GST manual, Pharmacia Biotech). No protease inhibitors were added to the lysis buffer to avoid inhibition of thrombin activity. The fusion protein was concentrated using dialysis buffer A (100 mM NaCl, 50 mM Tris-HCl, pH 8.0) followed by dialysis buffer B (50 mM NaCl, 20 mM HEPES-KOH, 20% glycerol, 10% polyethyleneglycol (PEG)) at 4°C as described in the GST manual (Pharmacia Biotech). 200 µg (100 µl) of the fusion protein was mixed with 900 µl of thrombin buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM MgCl2, 2.5 mM CaCl2, 1 mM DTT) and two units of thrombin (from bovine plasma, 600 NIH units/mg, Sigma) and rotated in the cold room overnight. Samples were taken at zero hours, 0.5 h, 1 h, 2 h, 4 h and 24 h to analyze on SDS-PAGE acrylamide gels and determine the efficiency of thrombin digestion with time. To purify the FEM-2 antibody a method of Takawa and Kagawa was used (K. Takawa and H. Kagawa, Okayama University, Japan, personal communication). 1.2 mg of fusion protein were digested for 24 h with thrombin and separated on a large 8% SDS-PAGE gel. The proteins were transferred to a nitrocellulose membrane. The membrane was stained with Ponceau S to visualize the protein (Harlow and Lane, 1988; Sambrook *et al.*, 1989). A narrow strip was cut parallel to the direction of migration and analyzed by Western analysis. This helped in the localization of the band containing the cleaned FEM-2 protein. The band was excised and incubated overnight with 1 ml of antiserum in the cold room. The strip was rinse with five changes of Phosphate-buffered saline (PBS), pH 7.4, and subsequently overlaid with 300 μl of 0.2 M glycine-HCl, pH 2.0, for 10 min to elute the antibody. The elute was adjusted to neutral pH by the addition of 48 μl of 1 M Tris, pH 8.0. The purified antibody was frozen in aliquots.

2.10 Western Analysis

Protein extracts of *C. elegans* were prepared as described by Goetnick and Waterston (1994). Briefly, well-fed worms of appropriate genotype were washed off the plate with M9 buffer, washed several times and incubated in M9 for 30 minutes to one hour to remove the bacteria from the gut. The worms were then washed with 4% sucrose solution and frozen at -20°C until animals for all the different alleles of *fem-2* were collected. Worms were resuspended in an equal volume of buffer containing the following proteinase inhibitors: 2 mM EDTA, 1 mM EGTA, 40 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM PMSF, 10 mg/ml leupeptin, 1 μg/ ml pepstatin, 10 μg/ ml aprotinin and 10 μg/ ml TPCK. The sample was microwaved for 25 seconds and transferred immediately into boiling water bath for five minutes. Standard Bradford assays were performed to determine protein concentration in worm samples (Bradford, 1976). After addition of loading buffer, the samples were boiled for an additional 5 minutes, centrifuged at 13000 rpm with a desk top centrifuge and separated on a SDS-PAGE gel. Electroblotting of SDS-PAGE gels to nitrocellulose (Schleicher and Schuell) and Immobilon P (Millipore) was performed at room temperature for 1 h at 100 V in 5 mM Tris, 38 mM glycine (pH 8.3), 20% methanol as specified by the manufacturer (Trans-blot transfer cell, BioRad). Proteins transferred to the nitrocellulose membrane were visualized using Ponceau S staining as described by Sambrook *et al.* (1989) and Harlow and Lane (1988). Immunodetection was carried out using either alkaline phosphatase or ECL (Amersham Life science) detection systems. The nitrocellulose membrane was incubated in Phosphate-buffered saline (PBS) with 0.5% Tween-20 (volume/volume) containing 5% skim-milk powder (Lucerne) (weight/volume) at 4°C overnight. The membrane was incubated in the above solution containing anti- FEM-2 primary antibody (1: 400 to 1: 20000 dilution) for 2 h to overnight at 4°C, then washed four times for 5 to 10 minutes in PBST. For Alkaline Phosphatase detection the blots were incubated in PBST/ milk powder containing 1: 5000 dilution of goat anti-rabbit IgGs linked to alkaline phosphatase (Boehringer Mannheim) for 1 h to overnight and then washed as above. Blots were stained in alkaline phosphatase (AP) buffer (10 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$) containing 0.3 mg/ml nitroblue tetrazolium (NBT) and 0.2 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Boehringer Mannheim).

ECL immunodetection was carried out using the ECL kit according to the manufacturers specifications (ECL Western blotting protocols, Amersham Life Sciences). The following changes were made in the protocol. The primary antibody was used at a dilution of 1: 1500 with an incubation time of 2.5 h. The secondary antibody was used at a concentration of 1 in 10000 with an incubation period of 2.5 h. All washes and detection were carried out as specified by the manufacturer. Autoradiography was done using Fuji Medical X-ray film (Fuji, Japan). Exposures of fifteen seconds to five minutes were required.

2.11 Indirect Immunofluorescence of Whole Animals

Whole mount antibody staining of *C. elegans* was done using the freeze-crack method and the Hypochloride method described by Miller and Shakes (1995) with modifications suggested by Greg Mullen (personal communication). In summary frosted microscope slides were coated with poly-L-lysine and dried at 37°C for 30 minutes. Well-fed worms were washed off plates with EN buffer (10 mM EDTA (pH 7.4), 100 mM NaCl), washed several times with sucrose solution and resuspended in two volumes of liquid per volume of worms. 25-50 μl of resuspended worms obtained from one to three worm plates were spread on a coated slide, covered with a coverglass and frozen at -80°C for 15 minutes. Two coplin jars, one with 100% methanol and one with 100% acetone, were cooled to -20°C. The coverglass was pried off the slides and the slides were immediately transferred into the -20°C methanol jar for four minutes, followed by two minutes in -20°C acetone. Rehydration was carried out in a acetone series of exactly one minute in each of the following jars: 75%, 50% and 25% acetone, followed by TBS-Tween. Slides were then transferred to another Tween-TBS jar until ready to stain. Primary antibody dilutions of 1:100 to 1:1000 were tested. The best results were obtained with overnight incubations in a humidifying chamber at room temperature with primary antibody at a concentration of 1:400 of anti-FEM-2 antibody to Tween-BSA-TBS solution. The next day the slides were soaked for 2 h in Tween-TBS. All subsequent steps were carried out in the dark, because FITC is light sensitive. Secondary antibody solution was added to the slides at a concentration of 1:500 of anti-rabbit-FITC to Tween-BSA-TBS and the slides were incubated for 3 h in the humidifying chamber. The slides were then soaked for 2 h in Tween-TBS, covered with a antibleaching solution consisting of 90% glycerol and 2% DABCO and covered with a coverglass. Microscopy was carried out as described above using the FITC filter.

Five antibody staining controls were used in this experiment. Firstly, the primary antibody was left out, to check for non-specific binding of the secondary antibody. Secondly, the primary antibody was preblocked with thrombin digested GST/FEM-2 fusion protein, to eliminate staining. Thirdly, immunostaining was performed on the putative molecular null mutants of *fem-2*. Fourthly, both primary and secondary antibody were omitted, to investigate autofluorescence by itself and, lastly, monoclonal and polyclonal anti-UNC-52 antibody, for which the immunofluorescence staining pattern had previously been established, was used as control for my staining technique (Greg Mullen, personal communication; Miller and Shakes, 1995).

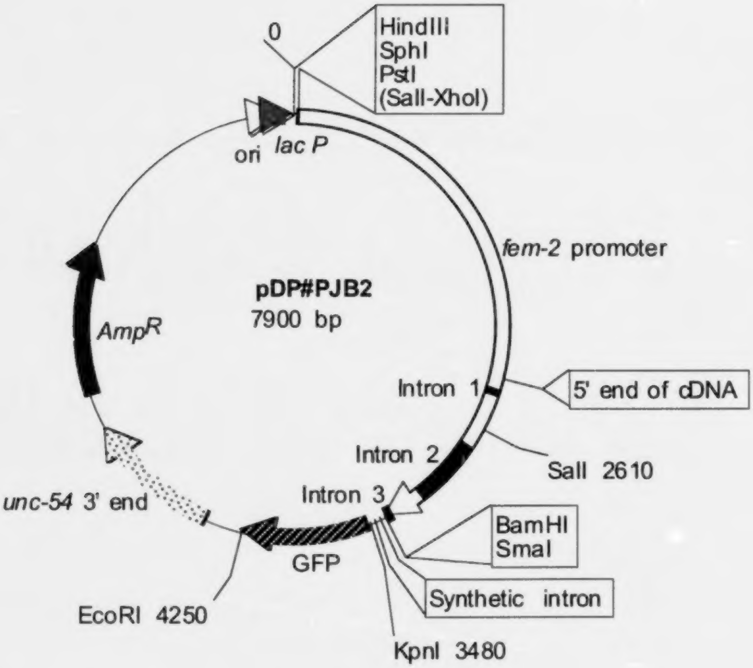
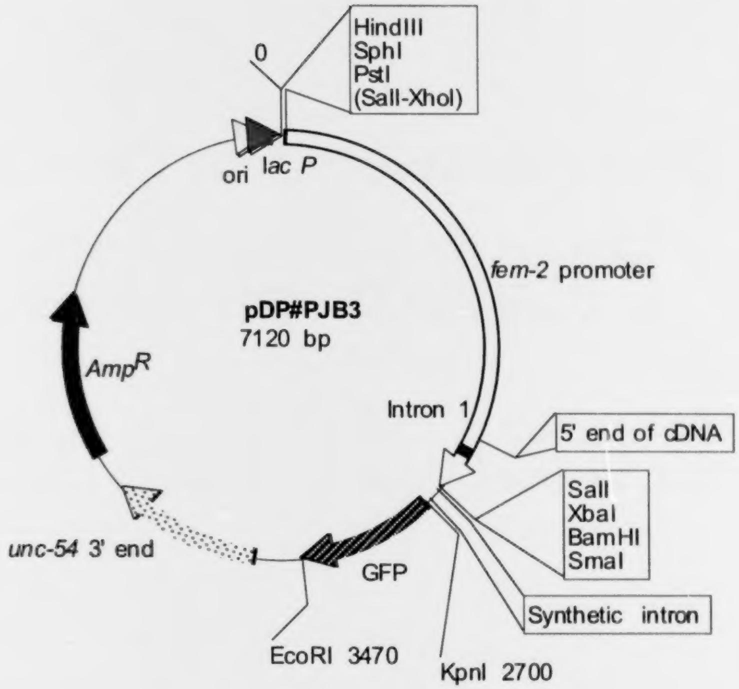

0
HindIII
SphI
PstI
(SalI-XhoI)
ori
lac P
fem-2 promoter
pDP#PJB2
7900 bp
AmpR
Intron 1
5' end of cDNA
Intron 2
SalI 2610
Intron 3
unc-54 3' end
BamHI
SmaI
GFP
Synthetic intron
EcoRI 4250
KpnI 3480

Figure 2-1. Vector pDP#PJB2 is based on TU#62, a green-fluorescent-protein (GFP) vector, which in turn is based on the lacZ fusion vector pPD22.04 (Fire *et al.*, 1990; Chalfie *et al.*, 1994). pDP#PJB2 has 2.3 kbp of *fem-2* upstream region and the first three exons and introns (1.1 kbp) of the gene inserted into TU#62.



0
HindIII
SphI
PstI
(SalI-XhoI)
ori
lac P
fem-2 promoter
pDP#PJB3
7120 bp
AmpR
Intron 1
5' end of cDNA
SalI
XbaI
BamHI
SmaI
unc-54 3' end
GFP
Synthetic intron
EcoRI 3470
KpnI 2700

Figure 2-2. Vector pDP#PJB3 is based on GFP vector TU#62 (Fire *et al.*, 1990; Chalfie *et al.*, 1994). pDP#PJB3 has 2.3 kbp of *fem-2* upstream region and the first exon, the first intron and part of the second exon (0.3 kbp) of the gene inserted into TU#62.

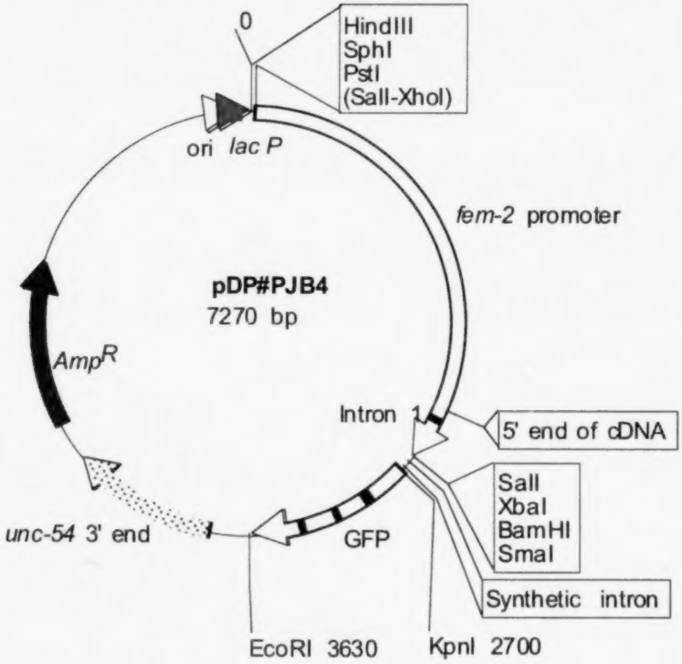

0
HindIII
SphI
PstI
(SalI-XhoI)
ori
lac P
fem-2 promoter
pDP#PJB4
7270 bp
Amp^R
Intron 1
5' end of cDNA
SalI
XbaI
BamHI
SmaI
Synthetic intron
unc-54 3' end
GFP
EcoRI 3630
KpnI 2700

Figure 2-3. Vector pDP#PJB4 is based on pPD95.69, a vector containing a modified GFP gene, which is reported to result in stronger and longer lasting fluorescence (A. Fire, personal communication). pDP#PJB4 contains 2.3 kbp of *fem-2* upstream region as well as the first exon, the first intron and part of the second exon (0.3 kbp).

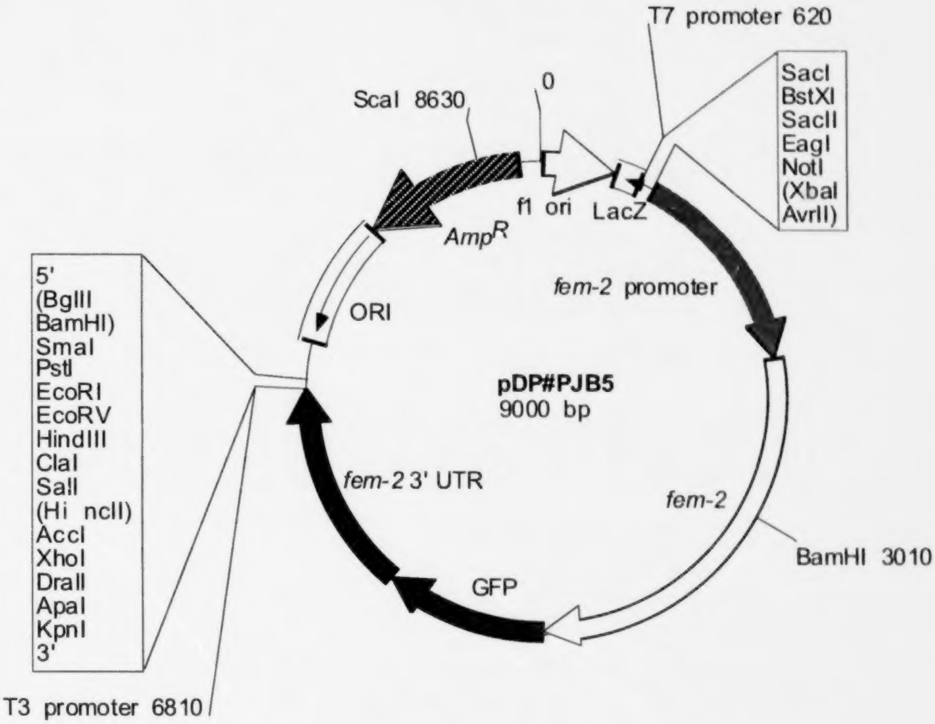

T7 promoter 620
SacI
BstXI
SacII
EagI
NotI
(XbaI
AvrII)
ScaI 8630
0
f1 ori
LacZ
AmpR
ORI
fem-2 promoter
pDP#PJB5
9000 bp
5'
(BglII
BamHI)
SmaI
PstI
EcoRI
EcoRV
HindIII
ClaI
SalI
(HincII)
AccI
XhoI
DraII
ApaI
KpnI
3'
fem-2 3' UTR
fem-2
BamHI 3010
GFP
T3 promoter 6810

Figure 2-4. Vector pDP#PJB5 is based on pBluescript with an insertion encompassing 1.3 kbp of *fem-2* upstream region, the entire *fem-2* gene and the modified GFP gene described in the text. pDP#PJB5 was obtained from Dave Hansen and Morris Maduro (University of Alberta).

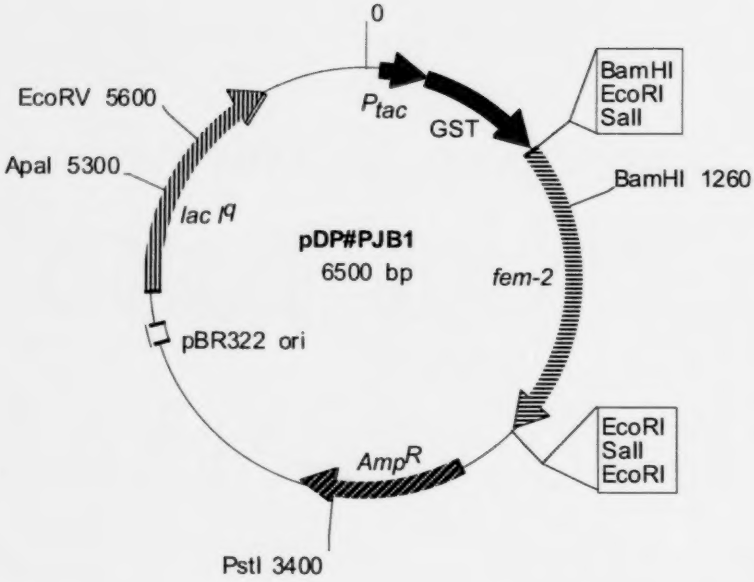

0
EcoRV 5600
ApaI 5300
P_{tac}
GST
BamHI
EcoRI
SalI
BamHI 1260
lac I^q
pDP#PJB1
6500 bp
fem-2
pBR322 ori
EcoRI
SalI
EcoRI
Amp^R
PstI 3400

Figure 2-5. Vector pDP#PJB1 was constructed by ligation of a *fem-2* cDNA fragment, which lacked 156 bp at the 5' end of the gene, into the pGEX-1λT vector (Pharmacia). Prior to ligation the pGEX-1λT vector was altered by the insertion of a SalI site into the EcoRI site of the polylinker. pDP#PJB1 was used to produce GST-FEM-2 fusion protein in *E. coli*.

Chapter 3 Results

3.1 *fem-2* cDNA Isolation

fem-2 was mapped to the left end of the *C. elegans* Linkage Group III using RFLP analysis (Pilgrim, 1993). This region was completely covered by Yeast Artificial Chromosomes and cosmid clones from the *C. elegans* physical mapping project (Coulson *et al.*, 1991) as well as by lambda clones (Pilgrim, 1993). Microinjections of cloned DNA fragments of this region of the physical map into *fem-2* mutant animals and subsequent tests for transformants with rescued phenotype (Materials and Methods) helped to narrow down the location of *fem-2* to a 15 kbp fragment (Pilgrim *et al.*, 1995). This 15 kbp fragment, DP#DBP003, was used as probe to screen a mixed stage *C. elegans* cDNA library (Barstead and Waterston, 1989) and a cDNA in the correct region was isolated (Pilgrim *et al.*, 1995).

I used two methods to determine whether the cDNA isolated corresponded to *fem-2*. First, the minimal rescuing region for *fem-2* was narrowed down to a 3 kbp genomic region which included this cDNA. Second, it was shown that a clone which previously caused rescue of the mutant phenotype would fail to do so after the deletion of a putative splice junction within *fem-2*.

3.1.1 Narrowing Down the Rescuing Region to 3 kbp

The putative *fem-2* cDNA as well as the corresponding genomic region were sequenced and restriction mapped with respect to DP#DBP003 (Pilgrim *et al.*, 1995). Clones that eliminated part of the remaining 12 kbp region were tested for their ability to rescue the *fem-2* mutant phenotype. Criteria and methods used for transformation rescue are described in Material and Methods.

Table 3-1 shows the clones tested, the number of transformants obtained and the number of transformed lines that retained the extrachromosomal construct for more than four generations. Figure 3-1 summarizes the rescuing results of the clones tested and shows their location on the physical map.

The results show that *fem-2* mutants could be rescued by both subclone DP#DBP026 and DB#DBP141. The new minimal rescuing region is defined by the 3 kbp overlap of these two subclones.

Even though the minimal rescuing region only included 239 bp of *fem-2* upstream region, the DB#DBP141 clone was able to completely rescue all phenotypes of *fem-2* null alleles (*b245 e2005*) when present as extrachromosomal arrays (Pilgrim *et al.*, 1995). This, however, does not necessarily indicate that the 239 bp upstream region contains the entire *fem-2* promoter. *fem-2* expression sufficient to rescue the mutant phenotype might be achieved by a high copy number of the rescuing clone in the extrachromosomal array (Mello *et al.*, 1991).

3.1.2 Subclone DP#DBP023ΔBamHI

Subclone DP#DBP023 has been shown to rescue the *fem-2* mutant phenotype (Pilgrim *et al.*, 1995). An altered version of this clone was constructed by deleting the central four base pairs of the BamHI site, which spans the boundary between intron 3 and exon 4 (Pilgrim *et al.*, 1995). The size and location of the deletion was confirmed by sequencing. The removal of the splice junction is predicted to result either in missplicing of the mRNA or, if splicing still occurred correctly, in a frameshift mutation introducing a stop codon and thus terminating the message shortly behind the deleted BamHI site. We would thus expect that the DP#DBP023 deletion clone would be unable to rescue the *fem-2(b245, ts)* mutant phenotype at the restrictive temperature. I obtained a total of 43 rollers from 11 different transgenic lines.

Transformation plasmids	Genotype injected	Number of worms injected	Number of rollers	Number of lines yielding rollers	Number of lines maintained	Rescue observed
DP#DBP023 + pRF4	*fem-2 (b245, ts)*	96	7	2	1	+
DP#DBP026 + pRF4	*fem-2 (b245, ts)*	129	29	12	4	+
DP#DBP141 + pRF4	*fem-2 (b245, ts)*	>80	14	5	2	+
DP#DBP023 ΔBamHI + pRF4	*fem-2 (b245, ts)*	245	43	11	1*	-

Table 3-1. The plasmids tested for their ability to rescue the *fem-2* mutant phenotype are shown. pRF4 contains the *Rol-6* reporter gene. Plasmid DP#DBP026, DP#DBP023 and DP#DBP141 rescued. DP#DBP023ΔBamHI, which contains a splice site deletion, failed to rescue. Moreover the later plasmid caused feminization in 70% to 96% of transgenic worms even at the permissive temperature, showing a dominant feminizing effect. * Transgenic lines could only be maintained by outcrossing with *him-8* males. Figure 3-1 shows the location of the plasmids in respect to the restriction map of the region around *fem-2*.

None of the lines showed rescue at the restrictive temperature. In contrast the 7 rollers obtained from 2 different lines carrying the DP#DBP023 transgene showed rescue of the mutant phenotype at 25°C.

In addition to demonstrating the inability of the deletion clone to rescue the mutant phenotype, I unexpectedly observed a dominant feminizing effect caused by the clone in transgenic animals at the permissive temperature. 70% to 96% of the transformed animals showed self sterility at 20°C. The remaining 4% to 30% had a much reduced brood size, consistent with a partial feminization. When crossed with males, self-sterile and partially feminized animals had a normal broodsize, indicating that oogenesis was normal and that the lack of offspring at 20°C was consistent with a specific defect in spermatogenesis, possibly due to feminization of the germline (FOG).

3.2 *fem-2* Promoter Controlled GFP Reporter Gene Expression

To investigate the developmental expression of *fem-2*, we chose to follow the expression of a reporter gene under the control of the *fem-2* promoter. The green fluorescent protein (GFP) was chosen as reporter, because it allows visualization in living animals. Four different reporter vectors (figures 2.1 to 2.4) were constructed and their expression patterns investigated in different genetic backgrounds of *C. elegans*. Details concerning the *fem-2* sequences and special features present in each of the vectors are described in the Materials and Methods section. Table 3-2 summarizes the transgenic lines obtained with the different constructs.

Transformation Plasmids	Genotype injected	Number of worms injected	Number of F1 rollers	Number of lines showing rollers	Number of transformed lines maintained past F4
pDP#PJB2+ pRF4	N2	34	107	19	5
pDP#PJB3+ pRF4	N2	54	77	7	4
pDP#PJB4+ pRF4	N2	116	85	6	5
pDP#PJB4+ pRF4	*him-8*	73	35	10	1
	him-8	Lines produced by crosses between *him-8* males and pDP#PJB4 transformed N2 hermaphrodites		4	0
pDP#PJB4+ pRF4	*fem-1 (hc17 ts)*	102	40	4	2
pDP#PJB5+ pRF4	*fem-2 (b245 ts)*	56	11	7	4

Table 3-2. Summary of the transgenic lines obtained by microinjection of various of the *fem-2* GFP-reporter gene constructs described in figures 2-1 to 2-4. pDP#PJB5 resulted in feminization of hermaphrodites and partial feminization of males at the permissive temperature. Transgenic lines containing this plasmid had to be maintained by outcrossing. One of the four transgenic lines containing pDP#PJB3 has the plasmid integrated into its genome.

3.2.1 pDP#PJB2 and pDP#PJB3

19 transgenic lines containing the pDP#PJB2 vector were obtained in a wildtype background. Of these only five were maintained past the F4 generation. None of the transgenic lines containing pDP#PJB2 showed any detectable amount of GFP expression. One possible reason for this may be that splicing of intron 3 does not occur, because the acceptor site immediately downstream of BamHI has been altered. BamHI, one of the sites used for directional cloning of the *fem-2* fragment into the GFP vector, spans the junction between intron 3 and exon 4. Alternatively, it could be that splicing occurs correctly, but the expression vector used in combination with the *fem-2* promoter might not result in sufficient amounts of GFP to be detected. *fem-2* might only be needed in small amounts and the promoter governing its production might be rather weak.

Because of the possibility of mis-splicing in pDP#PJB2, a new vector was constructed, pDP#PJB3, that used a SalI site in the second exon of the *fem-2* gene as cloning site. A sequence junction at the SalI site could not lead to missplicing. Seven transgenic lines containing pDP#PJB3 in a wildtype background were obtained, of which only four were maintained past the F4 generation. One of these lines was shown to be a spontaneous integrant of the transgenic array into the genome. This line showed a number of phenotypic defects including dumpiness, partial egg laying defect, misformation of the vulva, reduction of the ability to move and overall anatomical disorganization. Transgenic worms carrying the pDP#PJB3 plasmid showed only weak GFP expression. Expression was seen in the pharynx, the intestine and intestinal precursor cells in embryos and the vulva. In addition GFP was seen concentrated in areas which have not been correlated with specific structures. However, GFP expression in transgenic lines containing pDP#PJB3 were mostly limited to the integrated strain, which showed a large number of phenotypic defects. Since I could not exclude the possibility that the GFP expression pattern was a result of the integration site rather than the regulation of the reporter gene by the *fem-2* promoter, I only had limited trust in the accuracy of the expression pattern seen. The non integrated GFP expression vector under the control of the *fem-2* promoter might have resulted in a GFP signal that was too faint to detect. For that reason pDP#PJB4 was constructed with an improved GFP vector (pPD95.69) and its transgene expression was investigated in a wildtype background.

3.2.2 pDP#PJB4

Six lines of transgenic animals containing the pDP#PJB4 plasmid were obtained of which five were maintained past the F4 generation. The GFP expression in animals from all five lines correlated well. GFP was observed in hermaphrodites in all cells of the intestine and in the pharynx in all developmental stages. The earliest GFP staining was seen in intestinal precursor cells in the late stages of gastrulation. In a small number of worms (0.5%) GFP expression was seen in body wall muscles and some nerves in the head region. Whether this is ectopic or meaningful GFP expression cannot be determined at this time. However, the two roles of *fem-2* do not suggest any function for the gene in body wall muscles. This in combination with the very low percentage of worms expressing GFP at that location, makes me assume that the expression of GFP in body wall muscles is ectopic. GFP expression might also have occurred in other areas of transgenic worms carrying pDP#PJB4, but at a level too low to be detected. Figures 3-2 and 3-3 illustrate the GFP expression observed in hermaphrodites and embryos respectively.

In order to investigate the expression of the *fem-2* promoter reporter gene construct in males, ten transgenic *him-8* lines were constructed by direct injection with the pDP#PJB4 vector. Only one of these lines was maintained. Additional pDP#PJB4 carrying *him-8* lines were obtained by crossing transgenic N2 hermaphrodites with *him-8* males. The only structure in transgenic males, which weakly expressed GFP was one nerve close to the tail. In addition the outcrossed transgenic males would sometimes show GFP expression in their intestinal cells.

Having established the pattern of GFP expression in wildtype animals, I investigated whether a mutation in one of the other *fem* genes (*fem-1* or *fem-3*) would in some way influence the expression of the reporter construct. Four transgenic lines containing pDP#PJB4 in a *fem-1(hc17, ts)* background were constructed. Three of these lines were maintained past the F4 generation. The GFP expression patterns in transgenic *fem-1(hc17, ts)* animals was identical to the expression pattern observed in wildtype animals. Even though over 100 animals carrying *fem-3* alleles were injected, no transformants could be obtained and potential changes in *fem-2* controlled GFP expression patterns could not be investigated.

3.2.3 pDP#PJB5

One shortcoming of the GFP vectors pDP#PJB2-4 was that we could not be sure that the expression pattern seen actually corresponded to the location where the *fem-2* product would be needed. For this reason the pDP#PJB5 plasmid, containing the 1.3 kbp of the *fem-2* upstream region and the entire *fem-2* gene fused in frame to the GFP coding sequence, was constructed as described in Materials and Methods. The ability of pDP#PJB5 to rescue the mutant *fem-2* phenotype as well as the GFP expression pattern caused by the plasmid were investigated in a *fem-2(b245, ts)* background. The plasmid did not rescue the mutant phenotype at the restrictive temperature and moreover caused feminization of hermaphrodites and partial feminization of the male germline at the permissive temperature. Transgenic lines containing this plasmid had to be maintained by outcrossing to *him-8* males. A total of four outcrossed lines were maintained, all of which showed the same GFP expression pattern, but varied in their intensity of expression.

Despite the plasmids inability to rescue, strong GFP expression was observed in hermaphrodite as well as male animals. The GFP expression pattern included all areas of expression observed in transgenic lines carrying pDP#PJB4, but showed additional fainter GFP expression in new areas. In hermaphrodites very strong GFP expression could be observed in the pharynx and the intestine. In addition weaker GFP staining was seen in nerves of the head region, the vulva and the spermatheca. The hermaphrodite staining pattern is illustrated in figure 3-4. In males GFP expression was observed in the somatic gonad, the distal tip cells, sperm cells and the intestine. No clear staining of the nerve cell in the male tail observed in trangenic animals carrying the pDP#PJB4 construct could be seen. Figure 3-5 summarizes the GFP expression seen in males carrying the pDP#PJB5 construct.

3.3 FEM-2 Protein Expression Studies

3.3.1 The Making and Cleaning of Anti-FEM-2 Antibody

The GST-FEM-2 fusion protein expression vector, pDP#PJB1, was constructed by the insertion of *fem-2* coding region into pGEX-1λT (Materials and Methods). Conservation of the reading frame was confirmed by sequencing across the insertion junction in the plasmid.

Lysates of induced *E. coli* cells carrying the pDP#PJB1 vector and of induced *E. coli* cells carrying the pGEX-1λT vector were compared on Coomassie blue stained SDS-PAGE electrophoresis gels. *E. coli* cells carrying the pDP#PJB1 vector are expected to produce a GST/FEM-2 fusion protein, while *E. coli* cells carrying the pGEX-1λT vector are expected to produce GST protein alone. These expectations were met, as the protein band patterns of the two lysates confirmed. The band patterns were identical except for an additional band of 65 K seen in the lysate with the GST/FEM-2 fusion protein and a 27 K band seen in the lysate with GST. The sizes of the extra bands seen correspond in weight to the expected size of the GST/FEM-2 fusion protein and GST protein respectively. Lysates from uninduced *E. coli* cells showed neither band. This confirmed that the new band seen in lysates of *E. coli* carrying pDP#PJB1 corresponded to the GST/FEM-2 fusion protein, that the inserted *fem-2* coding sequence was in frame with the GST sequence, that the fusion protein was not stored in inclusion bodies and that fusion protein could be produced in sufficient amounts for antibody production.

Fusion protein for antibody production in rabbits was produced, isolated and prepared as described in Materials and Methods. Prebleed serum as well as serum obtained from the first, second and final bleed were tested for cross reaction on Western blots of worm and *E. coli* extracts. The prebleed serum of one of the two rabbit used (4W6) was very clean, showing hardly any cross reacting bands in worm as well as *E. coli* extracts. The prebleed of the other rabbit (4W7) used showed considerably more cross reacting bands, some of them in the same size range as was predicted for the FEM-2 protein bands. For that reason serum of rabbit 4W7 was not used for any of the later FEM-2 expression experiments. The first, second and finals bleed sera of both rabbits were tested for their ability to cross react with FEM-2 or GST/FEM-2 on Western blots of worm and *E. coli* extracts. A 51 K band in lanes containing worm extract and a 65 K band in lanes containing *E. coli* extracts could be detected (data not shown). These sizes correspond to the expected sizes for the FEM-2 and the fusion protein respectively. In addition an increase in band intensity could be detected when comparing Western blots tested with first, second and final bleed serum. Because the prebleed of rabbit 4W6 showed the least background bands and the final bleeds of both rabbits gave the strongest bands corresponding in size to FEM-2, only the final bleed serum of the rabbit 4W6 was used for all further experiments. However a considerable number of background bands still remained on the Western blots treated with final bleed serum of rabbit 4W6. In order to reduce cross reaction of the serum to proteins other than FEM-2 the serum was twice precipitated with ammonium sulfate.

To further purify the anti-FEM-2 antibody, a GST/FEM-2 protein containing the full length version of FEM-2 was isolated from an induced *E. coli* culture.

The vector containing the full length FEM-2 fusion protein was obtained from Dave Hansen (University of Alberta). The isolated protein was digested for varying length of time with thrombin and the resultant samples were separated on a SDS-PAGE gel. Figure 3-6 illustrates that 24 h of thrombin treatment resulted in almost complete cleavage of the fusion protein into its GST and FEM-2 portions. Cleaved fusion protein was separated on a SDS-PAGE gel, transferred to a nitrocellulose membrane by Western blotting and stained with Ponceau S. The band corresponding to the full length FEM-2 protein was used in strip elution to further purify the obtained antiserum (Materials and Methods). The specificity of antiserum obtained after this step was tested on Western blots of worm extracts and shown to have a strong reduction in number and intensity of background bands (data not shown).

3.3.2 Antibody Specificity Testing and Expression Studies of FEM-2 Tested by Western Blotting

Worm lysates were prepared from N2, *fem-2(b245, 25°C)*, *fem-2(q117, 25°C)*, *fem-2(e2102)*, *fem-2(e2105)* and *him-8(e1498)* animals to analyze FEM-2 expression on Western blots. To correctly identify bands corresponding to FEM-2 or an altered FEM-2 product the following criteria were used. First I looked for a band of 51 K, the predicted size of the full length FEM-2. Second, the band should not appear on Westerns, where the anti-FEM-2-primary antibody solution was preblocked overnight with 200 μg of batch purified GST-FEM-2 (full length) fusion protein before addition to the Western blot. Lastly the putative FEM-2 band should be absent in null mutants.

As can be seen in figure 3-7a, a band of the predicted size was observed in extracts of wildtype, *him-8(e1489)*, *fem-2(b245, ts)* and *fem-2(q117, ts)* worms. In addition a band of approximately 27 K could be seen in all lysates. A second faint band slightly larger than the expected 51 K band could also be seen in all lysates. To verify the identity of the bands observed, a

Western blot was probed with preblocked anti-FEM-2 primary antibody. Figure 3-7b shows the altered band pattern observed under those conditions. The predicted 51 K band as well as the weaker 27 K band disappear completely under these conditions, indicating that they correspond to FEM-2 or altered FEM-2 products. The faint band, which is slightly larger than 51 K, is still visible under preblocked conditions. This band therefore most likely represents a protein that is slightly larger than FEM-2 and cross-reacts with the anti-FEM-2 antiserum. Similar cross-reacting, uncleanable bands on Westerns tested with polyclonal antibodies have previously been reported (Hoskins *et al.*, 1996). Extracts from the putative null mutants of *fem-2*, *e2102* and *e2105*, did not show a band of 51 K, supporting the idea that this band corresponds to the full length FEM-2 protein. However the 27 K band was very faintly visible in extracts of *fem-2(e2102)* and *fem-2(e2105)* animals. Overall it appears that the 51 K band corresponds to the full length FEM-2 product. The 27 K band seems to also correspond to a *fem-2* protein product. Since it is a lot smaller than the predicted size, since no alternative products are predicted, and since the ratio of upper to lower band varies slightly between lysate preparations and largely between different strains, the 27 K band might correspond to a degradation product of FEM-2. However since no other degradation products are seen on the Western, it is also formally possible that the 27 K band corresponds to an alternative form of FEM-2.

Figure 3-8 illustrates the difference in FEM-2 expression seen on Western blots between the two temperature sensitive alleles of *fem-2*, *b245* and *q117*. While the expression of *fem-2(b254, ts)* animals raised at the permissive or restrictive temperature is very similar to the expression pattern in wildtype worms, showing a strong band at 51 K and a weak band at 27 K, the pattern seen in lysates from *fem-2(q117, ts)* worms raised at restrictive temperature shows only a faint band at 51 K and a very pronounced band at 27 K. Lysates from *fem-2(q117, ts)* worms raised at the permissive temperature still show a strong band at 27 K, but also show a strong band at 51 K. It therefore appears that the mutation in *q117* somehow influences the amount of full length FEM-2 products made and that the effect is more pronounced at higher temperatures. The *b245* mutation does not seem to influence the amount of full length FEM-2 product made.

After determining the expression pattern of FEM-2 in the different alleles of *fem-2*, it was of interest to determine whether the expression pattern of FEM-2 was influenced by any other mutations in the sex determination pathway of *C. elegans*. Lysates of worms with mutations in *fem-1(hc17, ts)* or *fem-3(e1996)* were investigated on a Western blot probed with anti-FEM-2 antibody (figure 3-9). Lysates from worms carrying mutations in *fem-1(hc17, ts)* or *fem-3(e1996)* showed a strong 51 K band. However they also showed a number of smaller (around 45 K) bands. These bands might be degradation products of FEM-2. It is therefore possible that FEM-2 is degraded faster in cells carrying mutations in the sex determination pathway. However, these results are only preliminary and no firm conclusions can be drawn from these results at the moment.

Since FEM-2 is needed for male development, it is of interest when and where it is expressed not just in hermaphrodites, but also in males. For this reason lysates made exclusively from male worms were analyzed by Western blotting. As would be expected a weak band of 51 K could be seen (data not shown). However, since there is no simple way to isolate a large amount of males (they have to be hand-picked), it is difficult to isolate sufficient numbers of animals to get a clear Western blot. It might therefore be more appropriate to analyze the expression of FEM-2 in males by indirect immunostaining of whole mount preparations.

3.3.3 FEM-2 Homologs in Related Nematode Species

After having established that anti-FEM-2 antibody cross reacts specifically with FEM-2, I could use the antibody to identify cross reacting bands on Western blots containing worm extracts from related nematode species, which may allow the identification of putative FEM-2 homologs. This would give us an idea about the amount of conservation of the *fem-2* protein product and thereby of its importance in development. Two species were used in this study -- *C. briggsae*, which is most closely related to *C. elegans*, and *C. remanei*, a less closely related nematode species, which only has male and female, but not hermaphrodite, animals. Figure 3-10 shows the bands observed on Western blots of lysates of the wildtype worms of the three species. Two strongly cross reacting bands of around 97 K were identified in *C. briggsae*. These bands disappeared when the anti-FEM-2 primary antibody was preblocked with purified FEM-2 fusion protein prior to its application to the Western blot (data not shown). No cross reacting bands could be identified in *C. remanei*. These findings suggest that *C. elegans* and *C. briggsae* may possess FEM-2 homologs that are so closely related that they maintain sufficient structural similarities to cross react with the same antibody while differing in primary sequence enough to account for the differences in size.

3.3.4 Indirect Immunofluorescence of Whole Animals

To examine not just the presence or absence of FEM-2, but also its location in wildtype and mutant worms, I examined whole mount indirect immunofluorescence antibody stained animals. As a control to my procedure I obtained monoclonal and polyclonal anti-UNC-52 antibody from Gregory Mullen (University of British Columbia). These antibodies had previously been shown to stain the basement membranes associated with the body wall muscles in embryos of *C. elegans* (Miller and Shakes, 1995). Comparing the staining pattern I obtained with the published staining patterns, I could confirm that my freeze-cracking and staining method yielded the same results (data not shown).

Having thus established confidence in my technique, I could now use this method to investigate the FEM-2 expression pattern. Wildtype worms that were not treated with primary or secondary antibody showed weak yellowish autofluorescence in the intestine. This established the amount and color of fluorescence seen without any antibody treatment. Wildtype worms stained only with secondary antibody showed a weak diffuse green background staining and the yellowish auto-fluorescence of the gut granules. Both controls helped to differentiate the background fluorescence from FEM-2 expression. In clear contrast to control animals, adult wildtype hermaphrodites stained with primary anti-FEM-2 antibody followed by secondary anti-rabbit-FITC antibody showed strong green fluorescence in the germline. The staining was concentrated in the cytoplasm of the oocytes. The intestine of adult worms showed very weak fluorescence. However, it was difficult to determine whether the level of fluorescence seen in the intestine exceeded background levels. The anti-FEM-2 staining seen in an adult wildtype hermaphrodites (figure 3-11a) can be contrasted to the staining seen in animals treated only with secondary antibody (figure 3-12). Strong staining was also observed in the cytoplasm of early embryos (up to 16 cell stage) (figure 3-11b).

Having established the FEM-2 expression pattern in wildtype worms, I investigated the pattern seen in mutants of *fem-2*. Adult *fem-2(q117, ts)* worms raised at the restrictive temperature showed germline staining, however the staining was mostly limited to the nucleus (figure 3-13a). Animals raised at the permissive temperature did not show nuclear localization of staining. A similar pattern was observed with adult *fem-2(b245, ts)* animals raised at the restrictive temperature (figure 3-13b). Again staining occurred in the germline and was localized to the nucleus. However, the staining pattern in *fem-2(b245)* worms was not as consistent as the one observed in *fem-2(q117)* animals, and sometimes showed little or no staining. It cannot be excluded that the lack of staining observed sometimes was due to experimental error, since the nuclear localization of the fluorescence in the germline of *fem-2(q117, ts)* and *fem-2(b245, ts)* animals raised at the restrictive temperature was repeatable.

No clear pattern of FEM-2 expression in the putative *fem-2* null mutants, *fem-2(e2102)* and *fem-2(e2105)* could be established. It would be expected that the animals would show a complete lack of FITC staining, however this expectation was not fully matched. Instead these animals would often showed stronger than usual background staining of all structures, showing a complete lack of localization of FITC staining. In addition fluorescence often appeared equally strong under different fluorescence filters, indicating that it was not FITC which produced the strong fluorescence. At this point I cannot exclude the possibility of any staining of FEM-2 or localization of staining in putative null mutants. However given the fact that no FEM-2 band could be detected on Western blots of null mutant lysates combined with the finding that the fluorescence of whole null mutants showed no localization and had the same intensity under different fluorescence filters, I believe that the staining observed in putative null mutants of *fem-2* is nonspecific background.

As an additional control for the FEM-2 expression seen in wildtype hermaphrodites I examined the staining pattern in worms which completely lack a germline (Beanan and Strome, 1992). A strain carrying a *glp-4* (*g*erm*l*ine *p*roliferation) mutation was stained with anti-FEM-2 antibody. Using those mutants no FITC staining above background was observed in any structures of the animals. This observation does not exclude that FITC staining occurs in some tissues, such as in the intestine, but if staining occurs, it has a level that is not detectable.

Lastly I investigated the FEM-2 expression pattern in *him-8(e1489)* males. Staining was seen in the germline and the intestine. However the staining patterns obtained were not very clear and thus the results concerning males are only preliminary.

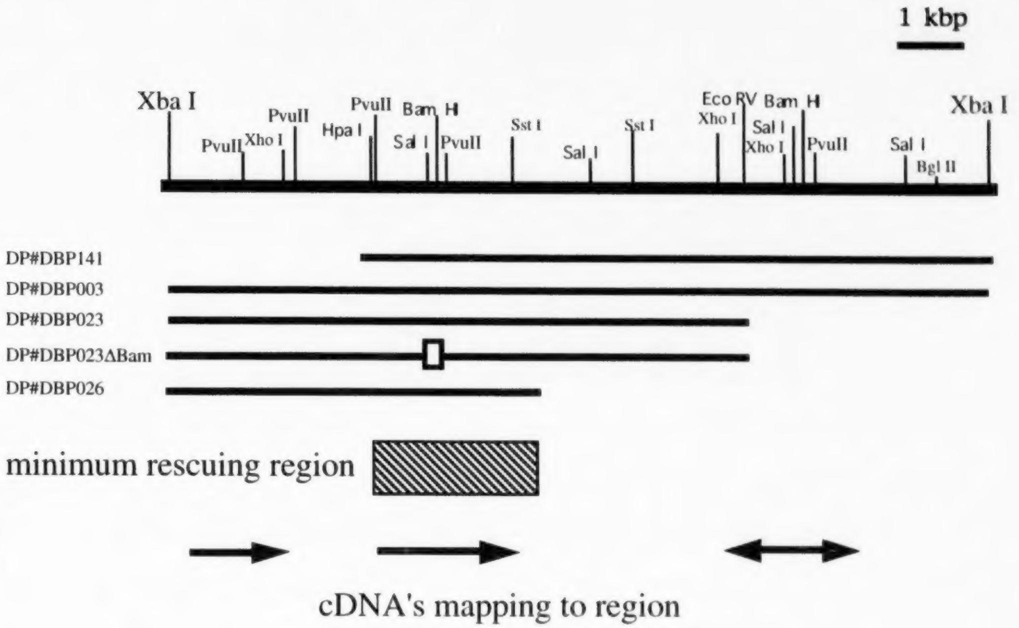
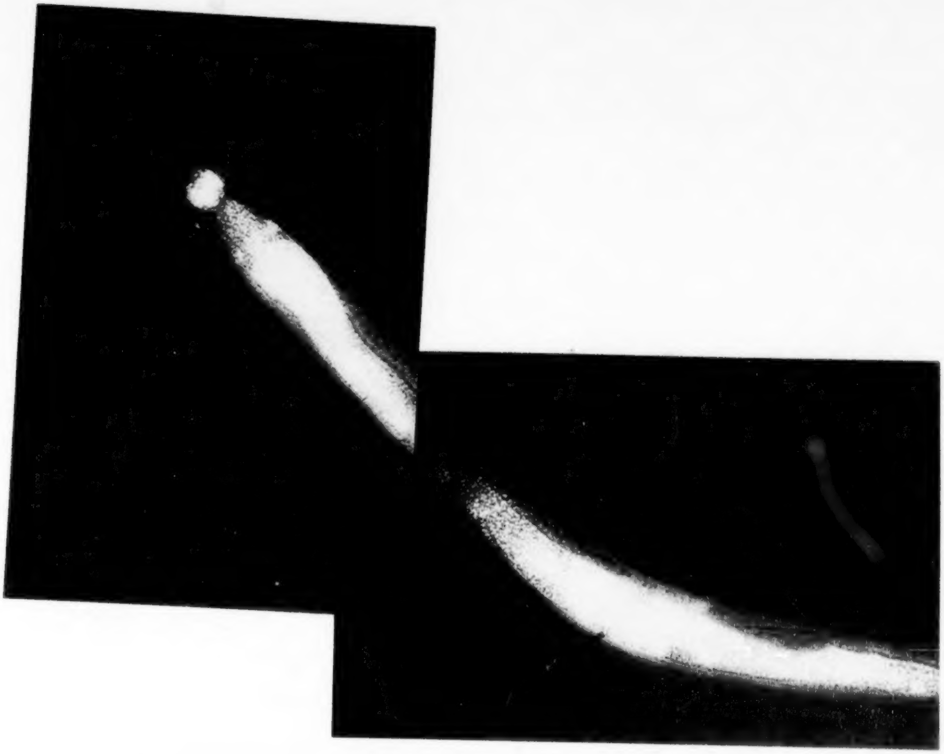

1 kbp
Xba I
PvuII
Xho I
PvuII
Hpa I
PvuII
Bam H
Sal I
PvuII
Sst I
Sal I
Sst I
Eco RV
Xho I
Bam H
Sal I
Xho I
PvuII
Sal I
Bgl II
Xba I
DP#DBP141
DP#DBP003
DP#DBP023
DP#DBP023ΔBam
DP#DBP026
minimum rescuing region
cDNA's mapping to region

Figure 3-1. Restriction map of the region around *fem-2*, aligned with the subclones tested for their ability to rescue the *fem-2* mutant phenotype. Subclones DP#DBP026, DP#DBP141, DP#DBP023 and DP#DBP003 were able to rescue, while DP#DBP023ΔBamHI was unable to do so. The overlap between DP#DBP026 and DP#DBP141 defined a new minimal rescuing region for *fem-2* of 3 kbp. DP#DBP023ΔBamHI was used to prove that clones with deletions in the *fem-2* coding region can no longer rescue the mutant phenotype. DP#DBP003 was the clone used to isolate *fem-2* cDNA from a cDNA library (Pilgrim *et al.*, 1995; Barstead and Waterston, 1989).

Figure 3-2. GFP expression in wildtype adult hermaphrodites carrying the reporter gene plasmid pDP#PJB4. (a) shows an animal photographed under fluorescent light with FITC filter and background illumination. GFP expression (yellow in this print) can be seen in almost all intestinal cells and in the pharynx. (b) shows the same worm photographed under Nomarski optics.

a.



b.

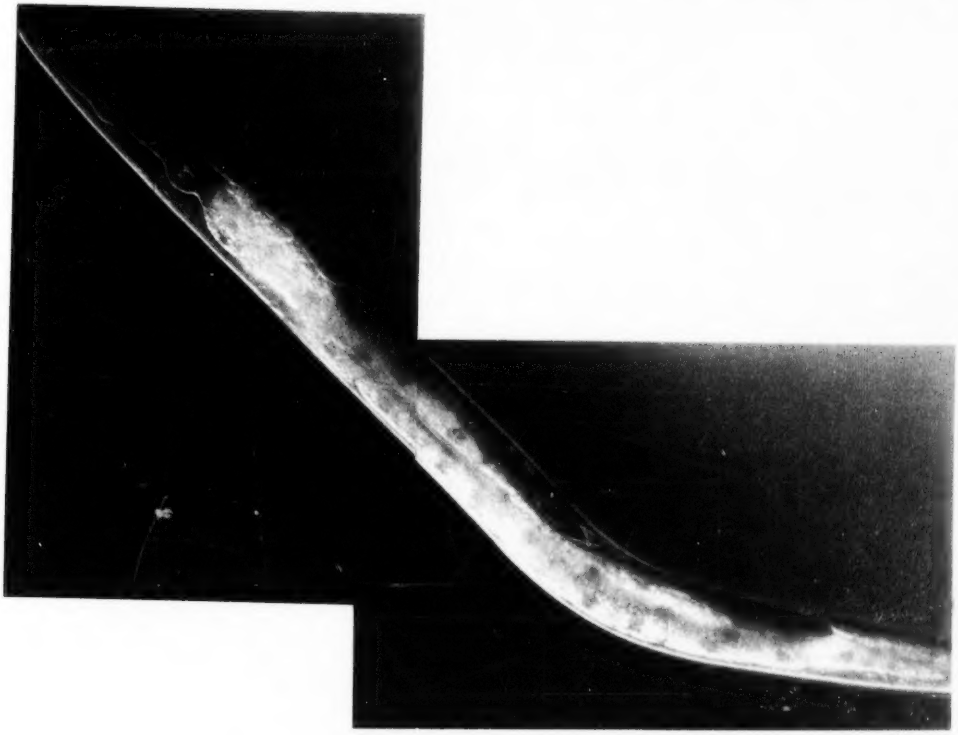
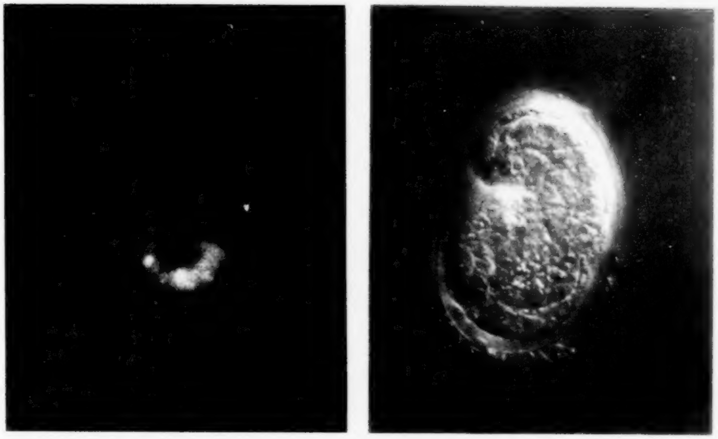


Figure 3-3. GFP expression in wildtype embryos carrying the reporter gene plasmid pDP#PJB4. (a) shows an early embryo photographed under a blue filter. GFP expression (white/blue in this print) can be seen in intestinal precursor cells. (b) shows a photograph of an embryo around the comma stage under Nomarski optics and fluorescent light with FITC filter. GFP expression (green) can be seen in the intestinal precursor cells. The two photographs are taken at different magnifications (1000X and 400X respectively). (c) shows an embryo around the time of hatching. GFP expression can be seen in almost all intestinal cells and in the pharynx.

a.

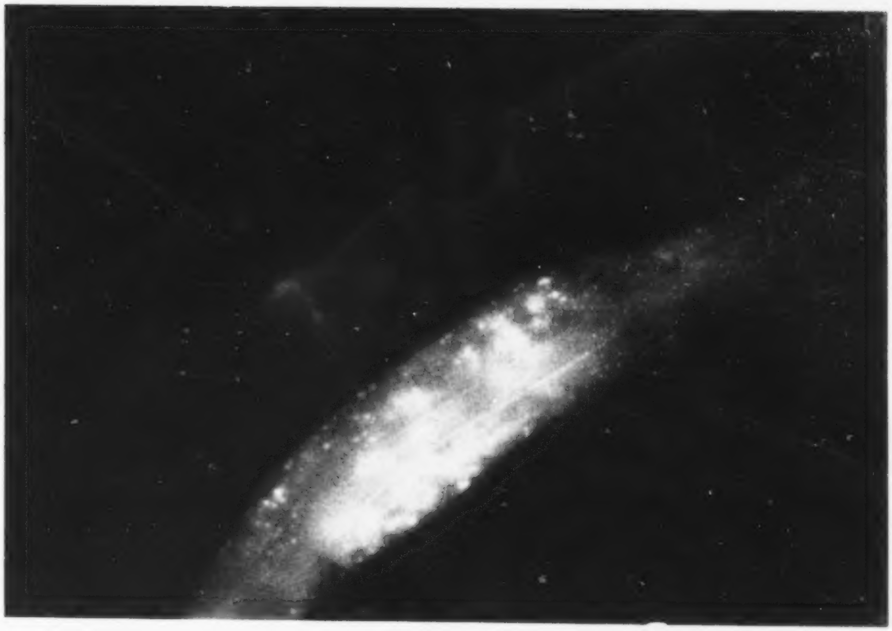
b.



c.

Figure 3-4. GFP expression in a wildtype L4 hermaphrodite carrying the reporter gene plasmid pDP#PJB5. (a) shows the midventral region. Dorsally the GFP expression in the intestinal cells can be seen (green) on top of the autofluorescence of the gut granules (yellow). Ventrally the vulva and the spermatheca can be seen to express GFP. (b) shows the head region of an adult hermaphrodite. GFP expression can be seen in the pharynx, the intestine and some nerves.

a.



b.

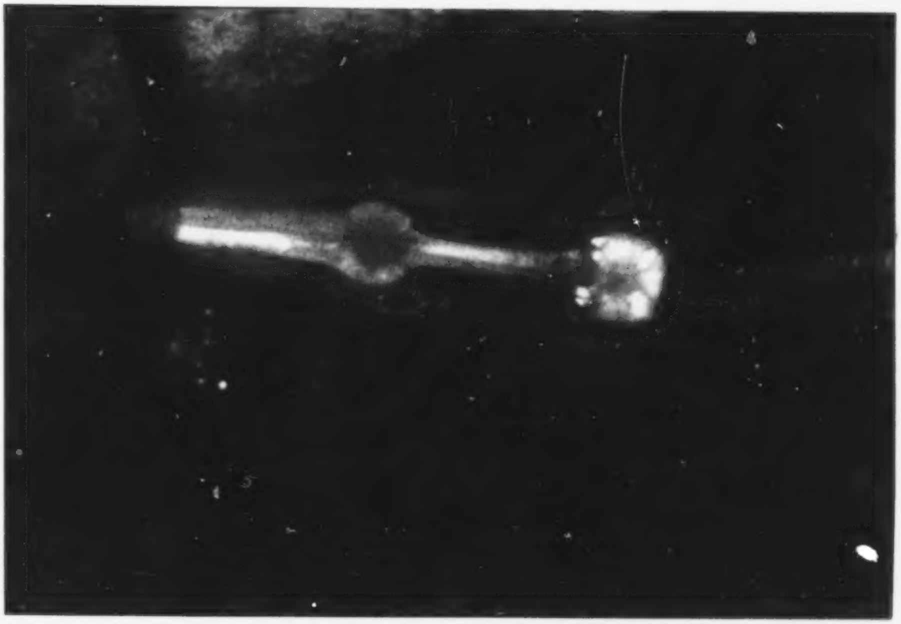
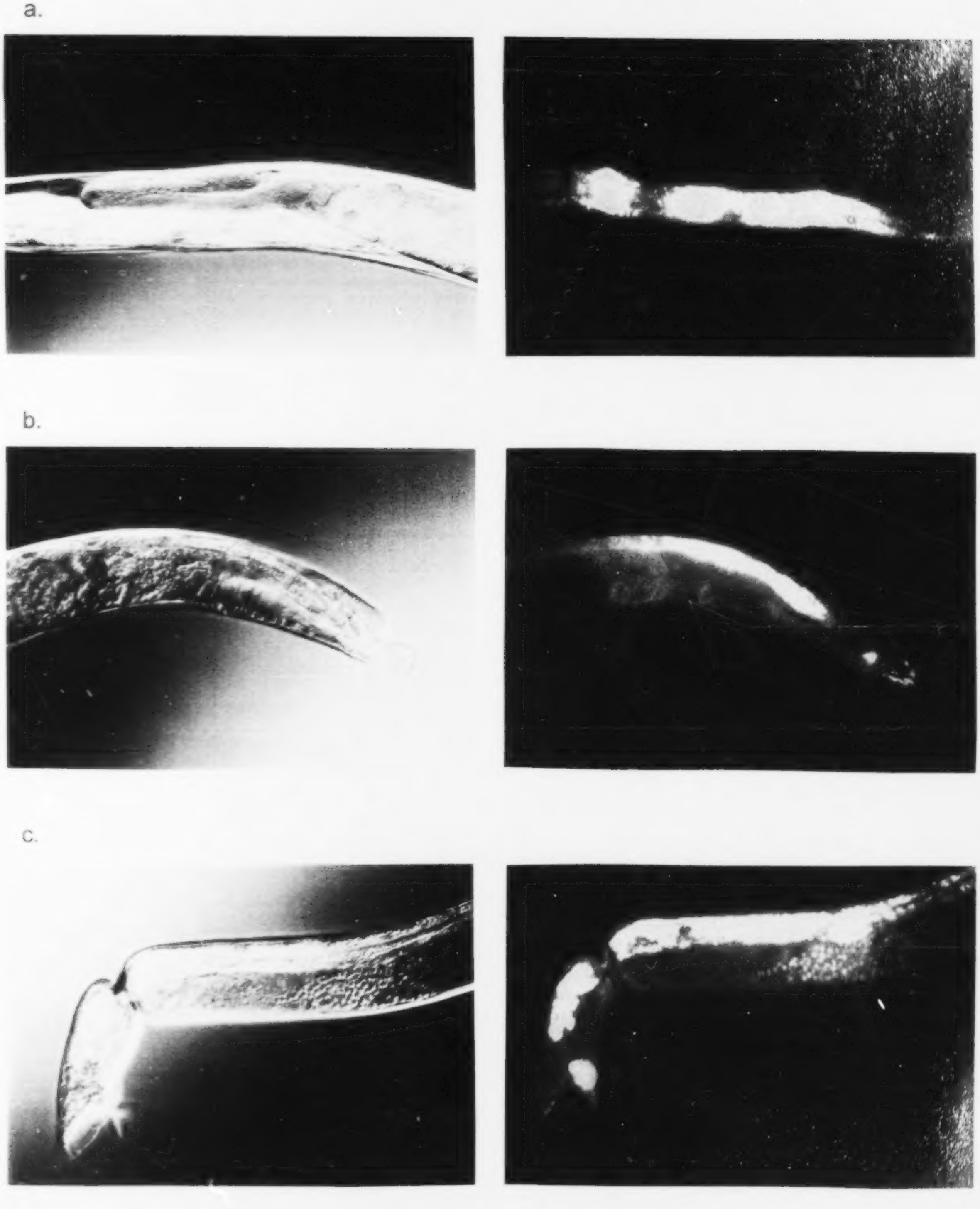


Figure 3-5. GFP expression in adult males carrying the reporter plasmid pDP#PJB5. The photographs on the left show the animals under Nomarski optics, the ones on the right show them under fluorescent light with FITC filter. In these pictures the yellow staining in the intestines is a mixture of autofluorescence from the gut granules and weak GFP expression. In the animals GFP expression (green) can be seen in (a) the DTCs at the distal tip of the gonad, (b) the proximal part of the somatic gonad and (c) in sperm cells.


a.
b.
c.

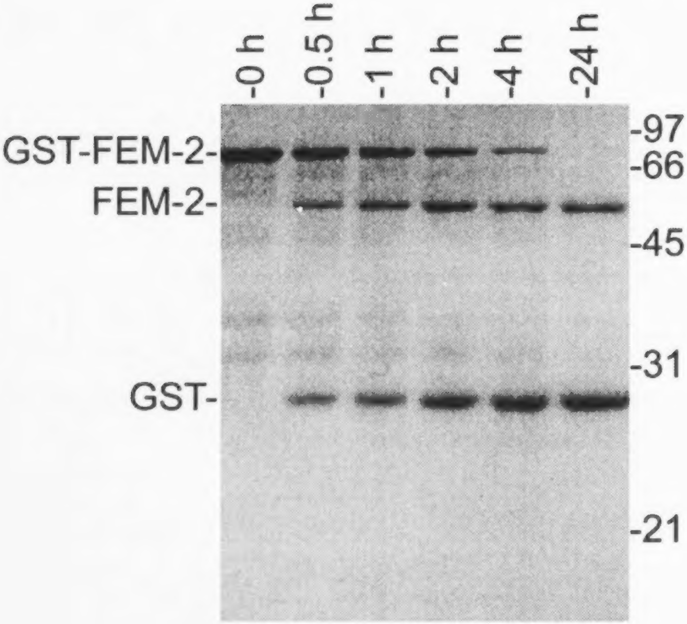
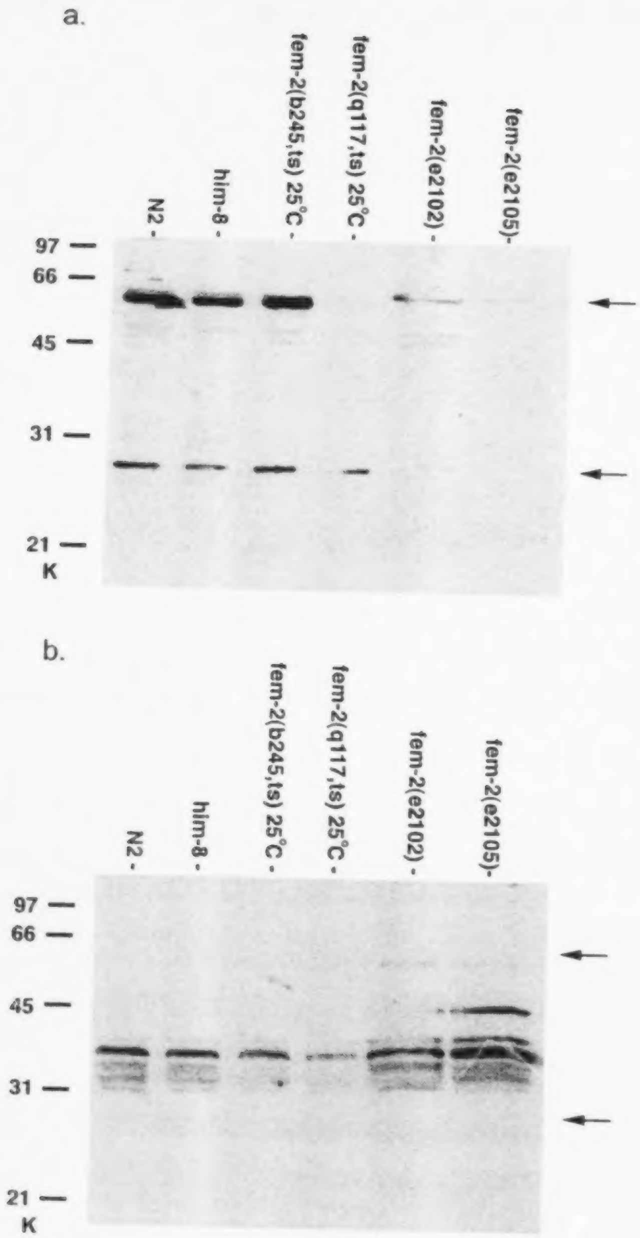

-0 h
-0.5 h
-1 h
-2 h
-4 h
-24 h
GST-FEM-2-
FEM-2-
GST-
-97
-66
-45
-31
-21

Figure 3-6. Coomasie blue stained SDS-PAGE electrophoresis gel of thrombin digested GST-FEM-2 fusion protein. 24 h of thrombin digestion resulted in almost complete cleavage. The FEM-2 portion was isolated and used for further cleaning of the anti-FEM-2 antibody.

Figure 3-7. (a) Western blot of worm extracts probed with anti-FEM-2 antibody. The 51 K band is the putative full length FEM-2 band. A second cross reacting band of 27 K can be seen. (b) Western Blot of Worm Extracts Probed with Preblocked Anti-FEM-2 Antibody. Both the 51 K and the 27 K band are completely blocked. Arrows label the position of the 51 K and the 27 K bands on both Western blots.


a.
N2
him-8
fem-2(b245,ts) 25°C
fem-2(q117,ts) 25°C
fem-2(e2102)
fem-2(e2105)
97
66
45
31
21
K
b.
N2
him-8
fem-2(b245,ts) 25°C
fem-2(q117,ts) 25°C
fem-2(e2102)
fem-2(e2105)
97
66
45
31
21
K

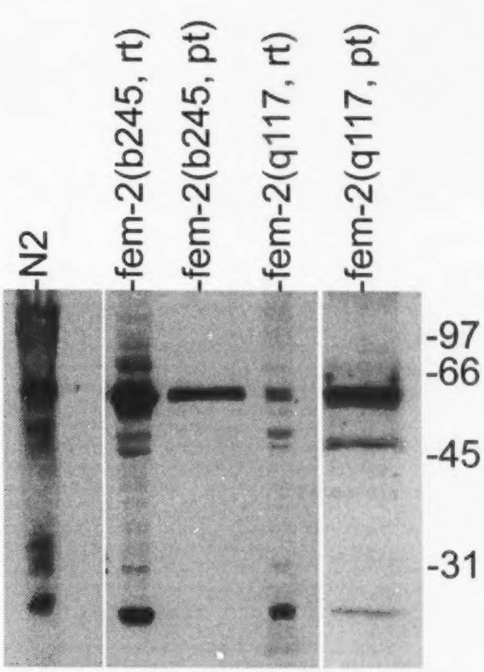

N2
fem-2(b245, rt)
fem-2(b245, pt)
fem-2(q117, rt)
fem-2(q117, pt)
-97
-66
-45
-31

Figure 3-8. Western blot of *fem-2(b245, ts)* and *fem-2(q117, ts)* worm extracts probed with anti-FEM-2 antibody. Animals were raised at the temperatures indicated. 25ºC is the restrictive and 20ºC is the permissive temperature. The band pattern for *fem-2(b245, ts)* extracts is the same at both temperatures, while the one for *fem-2(q117, ts)* extracts shows mildly reduced amounts of the 51 K putative full length FEM-2 band at permissive temperatures and a marked decrease of the 51 K band at the restrictive temperature.

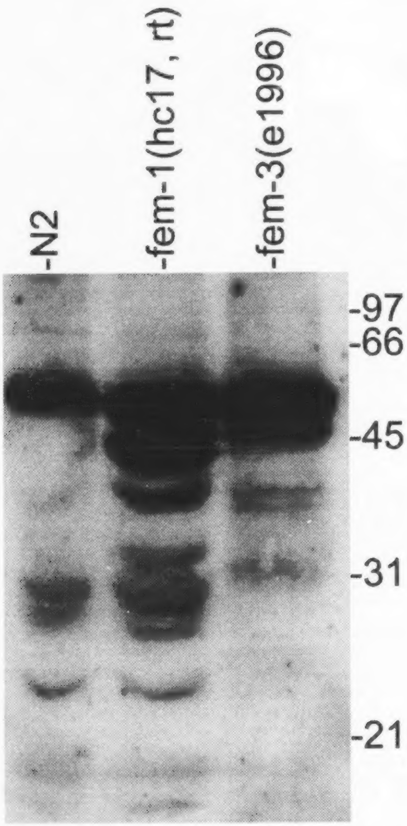

-N2
-fem-1(hc17, rt)
-fem-3(e1996)
-97
-66
-45
-31
-21

Figure 3-9. Western blot of worm extract from other sex determination gene mutants probed with anti-FEM-2 antibody.

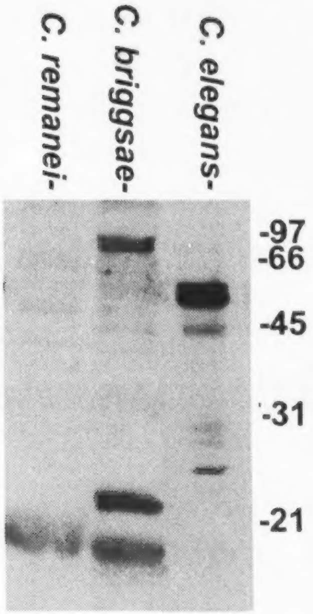
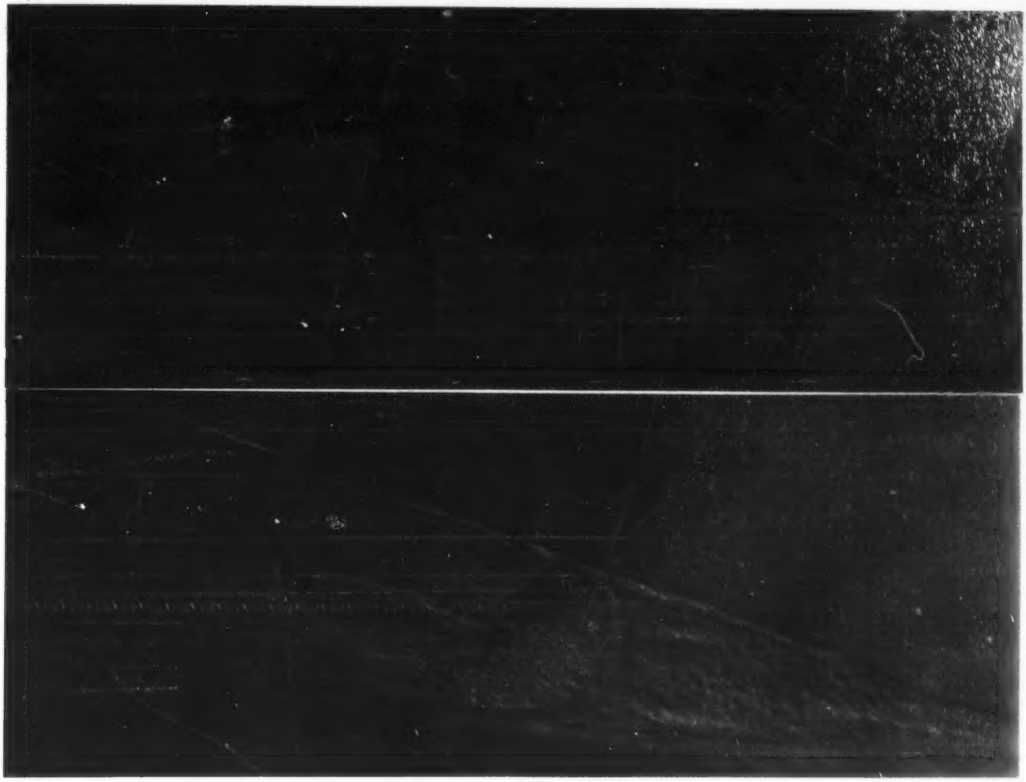

C. remanei-
C. briggsae-
C. elegans-
-97
-66
-45
-31
-21

Figure 3-10. Western blot of *C. elegans*, *C. briggsae* and *C. remanei* worm extracts probed with anti FEM-2 antibody. Extracts from *C. briggsae* showed two strongly cross reacting bands of approximately 97 K. No cross reacting bands could be detected in *C. remanei*.

Figure 3-11. Indirect immunofluorescent anti-FEM-2 antibody staining of (a) adult hermaphrodites and (b) embryos. Adult hermaphrodites (a) showed strong anti-FEM-2 staining in the germline, most prominently in the cytoplasm of the oocytes. Strong anti-FEM-2 staining can also be observed in the cytoplasm in early embryos (b).

a.



b.

Figure 3-12. Indirect immunofluorescent staining of an adult hermaphrodite using only secondary antibody. Diffuse green background staining can be seen in addition to the yellowish autofluorescence of the gut granules.

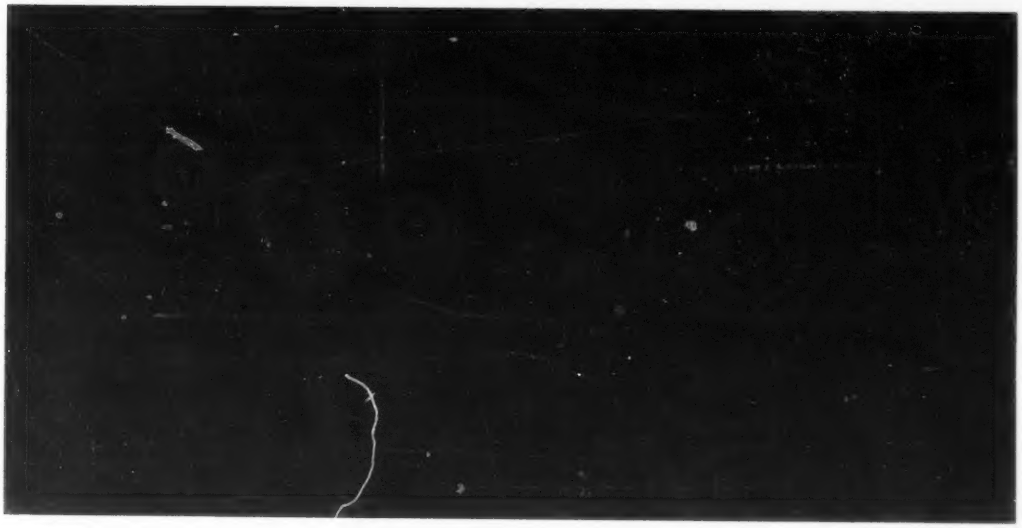
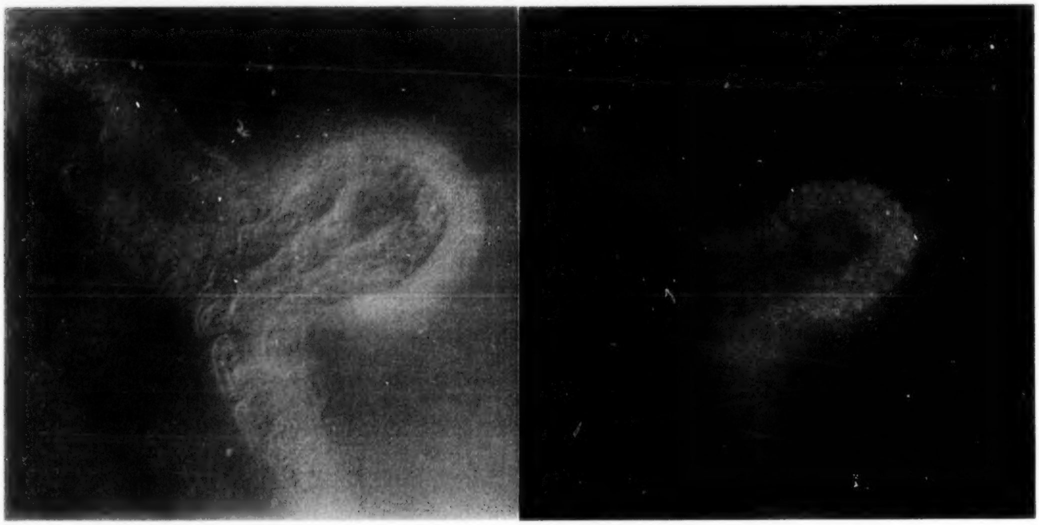


Figure 3-13. Indirect immunofluorescent anti-FEM-2 antibody staining of (a) *fem-2(q117, ts)* animals and (b) *fem-2 (b245, ts)* animals raised at the restrictive temperature. Anti-FEM-2 staining can be seen in the nuclei of the germline.

a.

b.



Chapter 4 Discussion

The sex determination pathway of *C. elegans* shows an amazing complexity. If we succeed in understanding the interactions in this pathway, we might gain new insights about signal transduction pathways in general, about the way pathways interact in determining the destiny of cells and about the development of an organism.

To put my project in context, it helps to look at a simplified scheme of the sex determination pathway. In this pathway TRA-2 has emerged as cell surface receptor while TRA-1 appears to be a transcriptional regulator. Genetic epistatic interactions predict that FEM-2 acts between TRA-2 and TRA-1, suggesting a role in the cytoplasm. Mutant analysis has shown that the *fem-2* gene product is a positive regulator of male fate in the germline and a negative regulator of female fate in somatic tissues. How does *fem-2* achieve its role? How does it interact with the other *fem* gene products? Which tissues and cells does it act in? Where does it act in the cell and when?

In order to elucidate the role of the *fem-2* in the sex determination pathway, we needed some means to follow its gene product. I have taken two approaches to follow the *fem-2* gene products. First, I investigated where a reporter gene is expressed under the regulation of the *fem-2* promoter, which might reveal the expression pattern of the *fem-2* transcript. Secondly I produced a polyclonal anti-FEM-2 antibody and used it to follow the expression of FEM-2 protein.

4.1 Confirmation of the Cloning of the *fem-2* cDNA

Before proceeding to study the expression of *fem-2* gene products we confirmed that the cDNA I had isolated earlier corresponded to the *fem-2* cDNA (Pilgrim *et al.,* 1995). Several lines of evidence suggest that we had indeed cloned the *fem-2* gene. First, two genomic subclones, which overlap by 3 kbp, can rescue the mutant phenotype of transgenic *fem-2* homozygous animals. Second, if one of the rescuing subclones is engineered to contain a 4 bp deletion in the putative *fem-2* gene, it can no longer rescue. Lastly, a minigene consisting of a genomic fragment containing 2.5 kbp of *fem-2* upstream sequence, exon 1, intron 1 and part of exon 2 ligated to a fragment from a cDNA clone containing the remainder of the exons and the poly (A) tail could rescue the mutant phenotype (Pilgrim *et al.*, 1995; McGregor, 1995). Taken together these findings confirm that transgenes containing the intact coding region of *fem-2* are necessary and sufficient to rescue the *fem-2* mutant phenotype in hermaphrodites and males. In further confirmation of this finding, sequencing of the mutant alleles of *fem-2* revealed base changes within the predicted coding region of *fem-2* in all alleles. In each case the base change would lead to an amino acid change (Pilgrim *et al.*, 1995). With these lines of evidence we were confident that we had cloned the *fem-2* locus and could proceed to investigate the expression patterns of the gene products.

4.2 GFP Expression Studies

In this study four different GFP vectors were used to follow the expression of GFP under the control of the *fem-2* promoter (Materials and Methods). Fluorescence could be observed in the following areas -- the intestinal cells and their precursors in hermaphrodites and sometimes in males, distal tip cells (DTC), the somatic gonad and sperm cells in XO animals, the spermatheca and the vulva in XX animals, the pharynx and some nerves in the head region in both sexes, and in a very small percentage of XX animals in some nerves outside the head region and in the body wall muscles. The putative identity of cells and tissues was determined using cell lineage charts and diagrams indicating the position of cells (Sulston and Horvitz, 1977; Sulston *et al.*, 1983).

Keeping the two biological roles of *fem-2* as well as the maternal effect exhibited by the gene in mind, we can classify the *fem-2* product into two classes. The first class consists of the maternally contributed *fem-2* product, *fem-2* product made by hermaphrodites to be contributed to developing oocytes. The second class of *fem-2* product consists of that made to specify spermatogenesis in the germline of animals of both sexes and to specify male soma development in XO animals.

4.2.1 GFP Expression in the Intestine

GFP expression was seen in the intestinal cells throughout all developmental stages. The intestine exhibits no anatomical sexual dimorphism, but a physiological difference between the two sexes has been reported (Kimble and Sharrock, 1983). In L4 and adult hermaphrodites yolk proteins are synthesized in the intestine, secreted into the body cavity and from there taken up by the gonad to reach the oocytes. This finding offers one possible explanation that links the reporter gene expression seen in the intestinal cells to the known biological role of *fem-2*. Since *fem-2* shows a maternal effect, there must be a maternal *fem-2* gene product contribution to the oocyte. It is therefore plausible that the intestine is the production site for the maternal contribution of *fem-2* product to the developing oocyte. Transcript production might start early in development in the intestinal cells and continue throughout the development of the worm to assure the presence of sufficient amounts of product for packaging into oocytes at all times.

Two other possible explanations for the expression of GFP in intestinal cell have been considered. The first one is based on the observation of a direct physical link in embryos of *C. elegans* between the germline precursor cells, Z2 and Z3, and the intestinal cells, INT5L and INT5R (Sulston *et al.*, 1983). This association ends around the time of hatching. Theoretically it is therefore possible that early intestinal cells directly transfer *fem-2* gene product to the germline precursor cells. However, this explanation seems unlikely, since it does not explain the high levels of *fem-2* expression seen throughout the rest of development.

The second explanation is based on previous reports of transgenic GFP reporter constructs that exhibited ectopic GFP fluorescence, which mostly occurred in the gut and pharynx (A. Fire and I. Hope, personal communication). Ectopic expression most often occurred when the promoter region governing GFP expression was too short. The possibility that GFP expression in transgenic animals is caused by ectopic expression of the reporter gene cannot be ruled out at this point, however several facts speak against this explanation. 0.3 kbp of *fem-2* upstream region have been found sufficient to fully rescue the mutant phenotype (Pilgrim *et al.*, 1995). The GFP reporter constructs, successfully used in this study, contain 2.3 kbp and 1.3 kbp of upstream region respectively. Both constructs provided very similar GFP expression patterns throughout the worm and identical expression patterns and intensity in the intestine. It therefore appears that the difference in upstream sequence did not influence the staining pattern in the intestine. However, this does still not rule out the possibility that 1.3 kbp and 2.3 kbp of *fem-2* upstream region contains too little of the promoter region to prevent ectopic expression or that ectopic expression is caused by the lack of control regions which lie downstream of the *fem-2* coding region and were not included in the reporter gene constructs.

Transgene expression in intestinal precursor cells in embryos, L1 and L2 animals has previously been reported using *fem-2*/lacZ expression constructs (Johnson, 1995). The results reported in the earlier study showed very strong mosaicism, a problem that is poorly understood and appears to affect some transgenic constructs more than others (A. Fire, personal communication). It therefore seems likely that expression also occurred in all intestinal cells. Mosaicism might also explain the lack of staining in animals older than L2.

In summary it seems most likely that *fem-2* gene product is produced in intestinal cells and packaged into the developing oocytes in a process similar to the production and packaging of yolk proteins. One observation that might initially seem in contradiction to the suggested model is the presence of reporter gene expression in the male intestine. However, here FEM-2 is likely to have a different function. FEM-2 is predicted to be present and active in all dimorphic cells in males to suppress TRA-1 activity. In the male intestine FEM-2 has to be active to suppress TRA-1 and thus the production of yolk proteins.

4.2.2 GFP Expression in the Somatic Gonad, the Distal Tip Cells and Sperm Cells of Males

The expression of GFP in the somatic gonad, the distal tip cells and the sperm cells in adult males together with the biological role of *fem-2* suggest several possible explanations for the staining pattern seen. The *fem* genes are involved in two decisions in males, they influence the production of sperm rather than oocytes in the germline and they influence the development of the male soma.

GFP expression was observed in the DTCs, the proximal part of the somatic gonad and sperm cells of XO animals (for reference of anatomy see figure 1-1). Staining of DTC cells had previously been reported in experiments that used a *fem-2* promoter/lacZ construct (Johnson, 1995). The simplest explanation for the staining in males is that *fem-2* product is required to specify male development in structures that have to develop a male fate. GFP expression was seen in the somatic gonad including the DTCs and in germ cells, that are to develop as sperm, structures that have to develop a male fate. Thus, *fem-2* gene product has to be expressed in the precursor cells of the somatic gonad to allow it to switch on male specific genes that will execute the cell fate as specified. This explains why *fem-2* product would be needed at some point during the development of those structures, but after the entire structure is already established, would there be a continuous need for *fem-2* product to specify fate? Temperature shift experiments suggest that this is not the case. The temperature sensitive period of *fem-2* for the development of the male somatic gonad has been found to be restricted to a short period in early larval development (Kimble *et al.*, 1984). Why then would we see GFP expression in adults? One possible explanation for this observation is that the reporter gene expression is not a direct mirror of the *fem-2* product expression. It is possible that *fem-2* gene product is degraded at a much faster rate than GFP, and that therefore the location of GFP expression, but not necessarily the time or the intensity, is indicative of *fem-2* gene product expression.

An alternative explanation for the GFP expression in the somatic gonad including the DTC cells in adult XO animals is the possibility of the existence of a sperm inducer. In addition to the observations described in the introduction, DTC ablation studies have revealed another important finding. Ablation of DTC cells in hermaphrodites up to the L3 stage causes all germ cells to produce sperm instead of oocytes. Furthermore it was shown that mutants of *fem-1* only made oocytes, even after the ablation of the DTCs. These results suggest that no committed precursor cell populations are set aside early in development. These findings might, however, be explained by the existence of sperm inducer. If a sperm inducer existed, its expression as well as the role of the DTC cells would be expected to be different in males and hermaphrodites. No GFP staining was observed in DTC cells of hermaphrodites, supporting the notion that the DTC cells in the two sexes differ in respect to gene expression and function. In males the sperm inducer might be continuously secreted by the somatic gonad and DTC cells. The *fem* genes might play a role in the secretion of the sperm inducer, by regulating some downstream genes. In XO animals where the DTC cells are ablated the amount of inducer might be reduced, but sufficient amounts are still released from the cells of the somatic gonad to specify sperm development. In hermaphrodites the hypothesis of a sperm inducer would require that the DTC cells would not release the inducer and furthermore might be involved in inhibiting its release from other cells of the somatic gonad. When the DTC cells are ablated in hermaphrodites other cells are no longer inhibited and start to release sperm inducer. The hypothesis that the *fem* genes are involved in the release of the sperm inducer might offer one explanation why *fem-1* mutants with deleted DTCs cells only produce oocytes. The second hypothesis to explain GFP expression in males is very complicated and requires many assumptions, I therefore favor the first explanation given above.

4.2.3 GFP Expression in the Spermatheca, Vulva and Other Areas of Females and in the Pharynx of Both Sexes

GFP expression was observed in the spermatheca and vulva of adult hermaphrodites. The spermatheca and the anchor cell, which controls vulva development, are part of the somatic gonad (Kimble and Hirsh, 1979). Both arise from the Z1 and Z4 cells, and thus share a common ancestry.

The spermatheca is used to store sperm, and *fem-2* is required to specify spermatogenesis. This leaves the possibility that the spermatheca somehow has to give off a product to specify spermatogenesis, such as a sperm inducer, as was suggested above. However the sperm stored in the structure are already completely specified and if a factor was given off to the developing gametes to specify sperm production it would seem more likely that it would be given off by a structure more distal in the somatic gonad, at the place where sperm cell maturation occurs.

Even though the spermatheca and the anchor cell, which is a precursor to the vulva, share the same ancestry and both form part of the somatic gonad, they have very little else in common. The vulva is not connected in any obvious way to functions of FEM-2. It is therefore difficult to explain the reporter gene expression seen in the vulva, and at this point I cannot make any reasonable suggestion in respect to its function.

The pharynx was one of the structures which showed the most prominent GFP fluorescence in both sexes. In addition to the pharynx a number of nerve cells in the head region showed reporter gene expression. No sexually dimorphic cells have been identified in the head region outside of the nerve ring, nor have any links been established between the head region, particularly the pharynx, and male somatic development or spermatogenesis (Sulston and Horvitz, 1977). The lack of any connection between the biological role of *fem-2* and the reporter gene expression seen in the pharynx of transgenic animals makes it difficult to explain the observed fluorescence. It is possible, but highly unlikely, that the *fem-2* gene product has a function besides its role in the sex determination pathway, and that the expression seen in the pharynx is related to that role. However, it is far more likely that the fluorescence seen in the pharynx is due to ectopic expression of GFP. This assumption is strengthened by previous GFP reporter gene studies in *C. elegans*, which showed ectopic GFP expression, most prominently in the pharynx (A. Fire, personal communication).

Ectopic expression is also the most plausible explanation for the expression of GFP in body wall muscles and some nerves in 0.5% of XX animals. The low percentage of animals exhibiting this expression pattern compared to the expression patterns described above and the lack of any correlation between the function of *fem-2* and the site of GFP expression, do not offer any reasonable alternative explanations.

4.2.4 Summary

From these results the picture is emerging that *fem-2* gene product expression can be classified into two classes-- product that will be contributed to the developing oocyte and product that is required directly in the developing animal to specify male fate. Expression of the first class of product can be seen in the intestine. Expression in the DTCs and somatic gonad in XO animals fall into the second class of product. It is not clear at this point whether *fem-2* is expressed in those structures to specify male development of the structures or whether the expression of *fem-2* is in some way related to the specification of spermatogenesis.

GFP expression was also observed in a number of unexplained areas, which I have largely discounted as ectopic expression. This reminds us that we have to take the shortcomings of the GFP reporter vectors into consideration. While for some genes investigated the GFP expression pattern correlated well with the known physiological expression pattern of the endogenous gene, other genes showed GFP expression patterns which showed incomplete correlation (A. Fire, personal communication). The most common problems included ectopic expression in the gut and pharynx, mosaic expression with only a subset of cells staining, and the failure of reporter genes to show expression in the embryonic, larval or adult germline as well as in pre-2 cell embryos (A. Fire, personal communication). It is therefore possible that physiological *fem-2* gene product expression occurs in the germline but cannot be detected using reporter genes. Likewise it is possible that some of the staining seen, for example in the pharynx or the intestine, represents ectopic expression and is thus meaningless. For this reason we should back our findings of transgene activity patterns by using additional techniques, such as in situ hybridization, to follow the *fem-2* transcript.

4.3 Feminization of the Germline

Unexpectedly a number of *fem-2* transgenes caused dominant feminization of the germline in XX and XO animals. This results in an apparent spermatogenesis defect causing self-sterility in XX animals and production of oocytes in XO animals. The soma of XO animals, including the somatic gonad, is not changed in any obvious way. The transgene constructs which caused the feminization of the germline phenotype in this study are DP#DBP023ΔBamHI, which was used to confirm the identity of the cDNA isolated, and DP#PJB5, the *fem-2* promoter/full length *fem-2*/GFP reporter vector. *fem-2(q117, ts)* worms carrying the DP#DBP023ΔBamHI transgene and *fem-2(b245, ts)* worms carrying the pDP#PJB5 vector were feminized at the restrictive and the permissive temperatures. In both cases the worms were fertile when crossed with males. These findings indicate that the transgenes acted in a dominant manner over the endogenous copies of *fem-2* and that they caused feminization of the cells which would normally become sperm, but that oogenesis remained unaffected. The same observations have previously been reported with the constructs pDP#TJ01, a lacZ reporter vector under the control of the *fem-2* promoter (Johnson, 1995). These observations might be the first step to separate the function of *fem-2* in germline development from its function in male somatic development.

A number of models can be suggested to explain these findings. It is possible that factors are required to bind to 5' upstream regions of *fem-2* for the gene to function normally. The presence of many extra copies of the 5' upstream region would titrate factors away from the wildtype copy, reducing expression from the genomic copy. However this explanation does not explain why other transgenes that carry equal amounts of upstream sequence do not have a feminizing effect. In addition the three vectors causing feminizing effect on the germline carried vastly different amounts of 5' sequence (3.5 kbp, 1.3 kbp and 2 kbp respectively).

A more plausible explanation is that the FEM-2 protein is composed of an interaction and an active domain. The amino terminus of the FEM-2 protein might bind other proteins at the sites where FEM-2 action is needed, while the carboxy terminus might contain the active domain, the region that carries out the enzymatic function of FEM-2. This separation of the protein into different domains is in agreement with the regions of sequence homology found between the PP2C class A phosphatases (Pilgrim *et al.*, 1995). In the cells carrying the transgenes the truncated FEM-2 proteins could bind to the sites where *fem-2* action is needed, thereby blocking them, but FEM-2 could not carry out its function, since it lacks the carboxy domain. This explanation still does not explain why feminization is seen in animals carrying the full length FEM-2/ GFP fusion protein, a protein which contains the suggested carboxy terminus. This later observation can be incorporated into the second explanation if one assumes that the site needed for enzymatic function is blocked by the GFP part of the fusion protein. To decipher what function different regions of FEM-2 have, we will have to carry out more detailed analysis of the effect of transgenes consisting of various parts of FEM-2 on the sex of the germline and the soma.

4.4 FEM-2 Protein Expression

4.4.1 Production of Anti-FEM-2 Antibody

The specificity of the antibody obtained was confirmed by the following findings. First, the prebleed serum of the rabbit, which yielded the antibody, did not cross react with any band on worm Western blots that was similar in size to the putative FEM-2 band. Second, a band of the size expected for full length FEM-2 (51 K) could be found in worm extracts. Third, this band could be blocked by preincubation of the anti-FEM-2 antibody with GST/FEM-2 fusion protein. Lastly, the two putative null mutants of *fem-2, e2105* and *2102*, lacked the putative FEM-2 bands. Together these findings were convincing evidence that the antibody isolated cross reacted with an epitope of FEM-2, was specific to FEM-2 and had a high enough titer to be used for further analysis of FEM-2 expression.

4.4.2 FEM-2 Expression Determined by Western Blotting

Two bands were identified on Western blots of worm lysates, one at 51 K, the expected size of full FEM-2 product, and one at 27 K. In wildtype extracts the 51 K band was considerably stronger than the 27 K band. This suggests that the 51 K band corresponds to full length FEM-2 protein, while the 27 K band corresponds to a degradation product or to a second form of *fem-2* protein product.

Worms carrying the temperature sensitive alleles *b245* and *q117* were analyzed under permissive and restrictive temperatures. While *b245* gave a pattern of bands identical to wildtype extracts under both temperatures, the pattern seen in *q117* extract varied considerably. This allows the hypothesis that the temperature sensitive mutation in *q117* affects the amount of full length FEM-2 protein, probably by causing structural instability. The increase in intensity in the 27 K band suggests either that this band corresponds to a degradation product of the full length product or that the mutation causes the preferential making of the shorter alternative product. It appear that the lack of full length FEM-2 contributes to the mutant phenotype seen in *q117* strains at the restrictive temperature. At the permissive temperature, even though a reduction in full length FEM-2 can be seen, enough product is made to fulfill the cellular function of FEM-2. The *b245* mutation does not influence the amount of full length FEM-2 product made and no difference can be seen between extracts of worms raised at permissive or restrictive temperatures. It therefore appears that the mutant phenotype is not caused by lack of full length FEM-2 product, but by a different mechanism. One plausible explanation is that at higher temperatures the instability caused by the mutation causes the protein to fold differently from the wildtype protein, therefore inactivating it.

Detection of FEM-2 on Western blots of worm extracts from mutants in *fem-1* or *fem-3* has shown that some mutations might cause less accumulation or faster degradation of the *fem-2* protein. The increased number of smaller cross reacting bands seen in extracts of the *fem* gene mutants, compared to wildtype worm extracts, might indicate that mutations in the *fem* genes speed degradation of FEM-2. In addition it is possible that they influence synthesis of FEM-2. At this point it is too soon to state anything conclusive. We will have to look at more alleles of *fem-1*, *fem-3* and the other sex determination genes, before we can determine whether a correlation exists between the mutations in other sex determination mutants and the change in FEM-2 expression.

Extracts of two closely related nematode species were tested with the anti FEM-2 antibody for any cross reacting bands. A doublet band of about 97 K was discovered in *C. briggsae*. Testing the Western blot with antibody preincubated with FEM-2 fusion protein, both bands disappeared, indicating that both are related to FEM-2. This finding indicates that *C. briggsae* has at least one, possibly two, proteins which are closely related to FEM-2. At this point we cannot determine whether the bands arise from two different genes or whether they are the products of one gene which is subject to posttranslational modifications. The discovery of cross reacting bands are our first step to the isolation of a potential FEM-2 homolog in *C. briggsae*. Conservation of FEM-2 across different species indicates that the protein and potentially the pathway in which it acts is used more widely.

No bands were seen to cross react with the anti-FEM-2 antibody in the *C. remanei* samples. There are two obvious explanations for this observation. First, there is a FEM-2 homolog in *C. remanei*, but it is too divergent to cross react with anti-FEM-2 antibody. Alternatively there may be no *fem-2* homolog in this species of nematode. This latter explanation would not be entirely unexpected, since the necessity of a sex determination pathway as complex as the one seen in *C. elegans* has previously been explained by the need to fine-tune the sex determination cascade to allow a brief period of sperm production in hermaphrodites (Hodgkin *et al.*, 1985). Thus a species lacking hermaphrodites might be able to specify sex by a much simpler cascade. Both *C. elegans and C. briggsae* have hermaphrodites and males as their two sexes, while *C. remanei* only has females and males. It is therefore possible that *C. remanei* specifies sex by a much simpler pathway.

4.4.3 FEM-2 Expression Determined by Indirect Immunofluorescent Antibody Staining

In adult wildtype hermaphrodites indirect anti-FEM-2 antibody staining was observed in the distal and proximal arms of the germline and was restricted to the cytoplasm of the germ cells. Weak staining was also seen in the intestine. In early embryos staining was seen in the cytoplasm of all cells. Keeping in mind the two classes of *fem-2* product, the maternally contributed *fem-2* product and the *fem-2* product required to specify spermatogenesis in the germline, the observed staining pattern is best explained as part of the former class. The staining observed seems to represent FEM-2 protein, which is packaged as maternal product into the developing oocytes. Assuming that the GFP staining in the intestine is authentic, we can note that *fem-2* transcript expression is abundant in the intestine, while *fem-2* protein expression is most prominent in the germline, but is also present at low levels in the intestine. These results can be explained by a mechanism, similar to the one described for yolk proteins, in which *fem-2* gets transcribed and translated in the intestine before it gets exported into the germline. The lack of prominent indirect antibody staining in the intestine can be explained by a difference in the timing, levels and rates of transcription, translation and protein export. GFP expression in the intestine has been seen from the embryonic to the adult stage, indicating that *fem-2* transcription occurs during the entire development. If the transcript is fairly stable a lot of transcript can accumulate. In addition the rate of *fem-2* transcription might be high, allowing for a large pool of *fem-2* mRNA to accumulate. Translation probably occurs later in development, since no staining could be seen in embryos or larvae. In addition it might also occur at a lower level. If the rate between translation and export of protein is about equal, than very little FEM-2 protein can accumulate in the intestine. If the protein is stable, however, which would be expected for a maternally contributed protein, a lot of FEM-2 can accumulate in the germline, its final destination.

Alternatively, we can explain the results by assuming that transcription occurs in the intestine and the transcript is exported into the germline before it gets translated. Since one drawback of reporter gene constructs is that they do not show reporter gene expression in the germline, the GFP studies described here cannot determine whether *fem-2* transcript is present in the germline. However this hypothesis does not explain why we do observe weak anti-FEM-2 antibody staining in the intestine.

A third possible explanation of the results seen is that the GFP observed in the intestine is ectopic expression and does not reflect where the *fem-2* transcript is present in the animals. It is thus possible that *fem-2* is transcribed and translated in the germline, but that we can only observe the protein product but not the transcript. Like the second hypothesis mentioned, this explanation cannot account for the weak anti-FEM-2 staining in the intestine. At this time we cannot exclude any of the three explanation. To differentiate between them, in situ hybridization experiments should be performed to obtain a complete cellular and developmental profile of the *fem-2* transcript.

We cannot determine with certainty whether the maternal contribution is in form of mRNA or protein. However looking at the early onset of anti-FEM-2 antibody staining in the germ cells and the amount of staining present, it seems likely that the maternal contribution to the developing oocyte is FEM-2 protein, not transcript. Maternal contribution in form of mRNA has previously been reported for *fem-3* (Barton *et al.*, 1987).

One very important conclusion that we can draw from our observations is that the phosphatase activity of FEM-2 must be regulated at a post-translational level. The FEM-2 product made in the hermaphrodite intestine has to be inactive to allow TRA-1 to direct yolk protein synthesis. FEM-2 in hermaphrodite embryos also has to be inactive to allow female soma development. Both observations suggest that the phosphatase activity of FEM-2 is regulated at a post-translational level.

It is somewhat surprising that anti-FEM-2 antibody staining was not observed in other parts of the worm, since the pattern described cannot explain how the protein fulfills its second role, the specification of spermatogenesis. It therefore seems likely that FEM-2 is expressed outside of the described areas, but that we failed to observe it. There are many possible reasons for this lack of observations. First, it is possible that FEM-2 expression occurs at a much lower level in the other areas and therefore is not detectable above background. Second, we would expect FEM-2 expression for spermatogenesis specification to occur around the L1 /L2 stage, not in adults. However I had problems to obtain good freeze cracking of worms at that stage and thus did not obtain good staining. Several improvements can be suggested to detect FEM-2 expression required for spermatogenesis in hermaphrodites. First, further investigation of FEM-2 expression with indirect immunofluorescence on the earlier developmental stages might elucidate the second role of FEM-2. Second, using confocal microscopy would be more sensitive and might thus reveal weaker staining areas.

In contrast to wildtype worms, animals which carry either *fem-2* temperature sensitive mutation, *q117* or *b245*, and which were raised at the restrictive temperature showed immunofluorescence which was confined to the nucleus of germ cells. Thus, even though the protein is expressed in the same set of cells it is localized to the nucleus rather than the cytoplasm. This indicates that the temperature sensitive mutations might result in a faulty subcellular localization of the FEM-2 product. This inappropriate localization of the FEM-2 protein might cause the mutant phenotype observed in the temperature sensitive mutants. In the case of the *q117* allele, the mutant phenotype might be caused by the combined effect of insufficient amounts of full length FEM-2 protein and inappropriate subcellular localization of the product.

One explanation for the inappropriate nuclear localization of FEM-2, is that FEM-2 acts in the nucleus and in the cytoplasm depending on the activity state it is in. The amino terminus, as was suggested above, might be used for binding other proteins, while another region of FEM-2 might be needed to regulate the protein-protein binding. If FEM-2 binds a nuclear protein, but cannot be dissociated from the protein because of a defect in its binding regulatory region, then the protein would be unable to leave the nucleus and perform its function in the cytoplasm.

Conclusion

This study has revealed some important findings and opened many new questions. It has confirmed the cloning of the *fem-2* locus, thus allowing further analysis of the gene. GFP expression studies have shown that the production site of maternal *fem-2* contribution is probably the intestine, from where the product gets exported to the germline for packaging. Furthermore reporter gene studies have shown *fem-2* expression in the somatic gonad, DTC and sperm cells of XO animals, linking these sites to the biological function of *fem-2*-- to specify spermatogenesis and to ensure male somatic development. The making of an anti-FEM-2 antibody created a powerful tool to analyze *fem-2* gene expression. Western analysis has identified two bands of 51 K and 27 K in wildtype worms, verifying the presence of FEM-2 protein and opening the possibility that a second FEM-2 product exists. It also verified that the phenotype observed in null mutants is probably caused by a lack of FEM-2 protein. Western analysis furthermore helped to identify a FEM-2 homolog in *C. briggsae,* but failed to do so in *C. remanei.* This is an indication that the sex determination pathway might be conserved in several nematode species and that conservation might depend on the existence of hermaphrodites in the species. Antibody studies proved, for the first time, that FEM-2 is present in the cytoplasm. Analysis of temperature sensitive mutants of *fem-2* gave an indication that FEM-2 can also be found in the nucleus, opening the possibility that it directly interacts with TRA-1. The timing and location of FEM-2 product in hermaphrodites and embryos, strongly suggests that FEM-2 is regulated at a post-translational level. The discovery of two transgenes with a dominant feminizing effect on the germline of XX and XO animals, together with the nuclear localization of FEM-2 in temperature sensitive mutants, has given us a starting point to further analyze the functions of different regions of FEM-2.

The study has also opened many questions. Where is FEM-2 protein expressed to fulfill its role in specifying spermatogenesis and male somatic development? What proteins does FEM-2 interact with? Is FEM-1, TRA-1 or a different protein its target protein? What function do the different regions of the FEM-2 protein have? Some of these questions can now be tackled using the antibodies made during this study.

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Appendix A

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The *C. elegans* Sex-determining Gene *fem-2* Encodes a Putative Protein Phosphatase

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The genetic and molecular analysis of genes involved in the regulation of sex determination in *Caenorhabditis elegans* suggests that the gene *fem-2* plays an important role in regulating a pathway transducing a non-cell–autonomous signal to a nuclear transcription factor. The wild-type *fem-2* gene was cloned by identifying sequences from the *C. elegans* physical map that could restore normal Fem-2 function to homozygous mutant *fem-2* transgenic animals. cDNA sequences mapping to the minimal rescuing region correspond to an open reading frame with a sequence similar to protein phosphatase 2C enzymes from systems as diverse as yeast, humans, and plants, but the alignments suggest that FEM-2 falls into a separate class of proteins than the canonical homologues. Several *fem-2* mutant alleles were sequenced, and the mutations are predicted to cause protein changes consistent with their observed phenotypes, such as missense mutations in conditional alleles, and a nonsense mutation in a predicted null allele. This is the first evidence implicating phosphorylation and/or dephosphorylation as a control mechanism in *C. elegans* sex determination

INTRODUCTION

The primary sex-determining signal in the nematode *Caenorhabditis elegans* is the ratio of sex chromosomes (**X**) to autosomes (**A**) (Nigon, 1951). Males have a single **X** chromosome per diploid cell (**X/A** ratio of 0.5) while hermaphrodites have two (**X/A** ratio of 1.0). The latter may be thought of as females that express a transient male phase (spermatogenesis) in their germ line before switching to the exclusive production of oocytes. Mutations have been isolated in *C. elegans* that cause animals to ignore the **X/A** ratio. Loss-of-function mutations in the *fem-1*, *-2*, or *-3* (feminization) genes cause XX and X0 animals to develop as females (the mutant hermaphrodites lose the ability to make sperm), while loss-of-function mutations in any of the three *tra* (transformer) genes cause XX animals to be masculinized. A genetic epistasis pathway has been proposed by Hodgkin using these, and other mutants, in which somatic sex determination in *C. elegans* is controlled by a hierarchy of negative regulatory interactions (Hodgkin, 1980, 1987), culminating in the gene *tra-1* (Figure 1). The genetic regulation of germline sex determination in *C. elegans* involves these, as well as germline specific genes (reviewed by Clifford *et al.*, 1994). Several genes linking the **X/A** ratio with both dosage compensation and sex determination have also been characterized (Villeneuve and Meyer, 1987; Miller *et al.*, 1988; Nusbaum and Meyer, 1989; DeLong *et al.*, 1993).

The *fem* genes have a central role in two decisions during development of *C. elegans*: whether to develop somatically as a male or female, and whether to make sperm or oocytes in the germ line (Kimble *et al.*, 1984; Hodgkin, 1986). Homozygous mutant alleles of the *fem* genes result in feminization of the animal, regardless of the karyotypic sex, implying that the wild-type function is to promote male development. Genetic evidence suggests that the *fems* act both as positive regulators of male germ cell development and as negative regulators of *tra-1*, a gene that promotes female somatic development. The *fem* gene products must be active in the male germ line and soma, and transiently active in the XX (hermaphrodite) germ line. The *fem* genes have been shown to be under different temporal control in the germ line and soma (Doniach and

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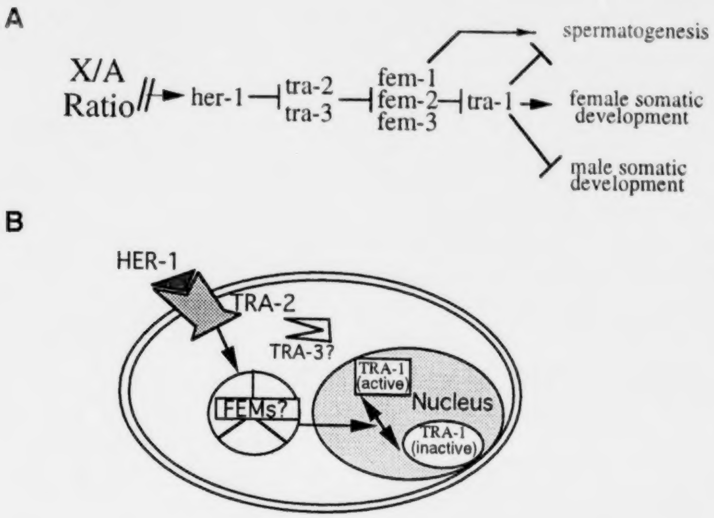

Figure 1. (A) Model of genetic interactions between the genes involved in somatic and germline sex determination in *C. elegans*. Only the genes downstream of *her-1* are illustrated, and genes affecting only the sex determination of the germ line (e.g. *fog* and *mog-1* genes [Schedl and Kimble, 1988; Graham and Kimble, 1993; Evans *et al.*, 1992]) have been omitted. Arrows indicate positive interactions, and bars indicate negative or repressive interactions. Adapted from Hodgkin, 1992. (B) Model for molecular interactions in the determination of somatic sex in *C. elegans*. Only gene products corresponding to the model in panel A are illustrated. See text for details. Adapted from Kuwabara and Kimble, 1992.

Hodgkin, 1984; Kimble *et al.*, 1984; Hodgkin, 1986), presumably due to negative regulation by the genes *tra-2* and *tra-3*. The activities of *tra-2* and *tra-3* are in turn repressed by *her-1*. Several of the genes in the pathway, including *fem-2*, demonstrate maternal rescue of the mutant phenotype (Doniach and Hodgkin, 1984; Kimble *et al.*, 1984), suggesting that either the mRNA or gene product is inherited through the female germ line. Because the *fem* genes act to promote male development, there must be control against inappropriate activity in XX animals. In contrast to *Drosophila*, where control of sex determination occurs at the level of mRNA splicing (reviewed in Hodgkin, 1990), evidence from *C. elegans* suggests control at both the transcriptional and translational levels (Doniach and Hodgkin, 1984; Rosenquist and Kimble, 1988; Evans *et al.*, 1992).

The genetic epistasis together with the molecular analyses of the genes have suggested that the cellular regulation of somatic sex determination in *C. elegans* involves a signal transduction pathway (Figure 1; reviewed by Kuwabara and Kimble, 1992) with control mechanisms that differ from other sex determination systems (Hodgkin, 1992). The *tra-1* and *-2*, *fem-1* and *-3*, and *her-1* genes have been cloned and characterized. HER-1 is predicted to be a secreted protein that acts non-cell autonomously (Hunter and Wood, 1992; Perry *et al.*, 1993). TRA-2 has the characteristics of a transmembrane receptor, possibly for HER-1 (Kuwabara *et al.*, 1992). The FEM-1 protein contains an ankyrin motif, suggesting that it is involved in protein-protein interaction (Spence *et al.*, 1990), whereas the predicted FEM-3 sequence is novel (Ahringer *et al.*, 1992). TRA-1 contains a zinc-finger DNA-binding domain and is probably a transcriptional regulator (Zarkower and Hodgkin, 1992). A complete molecular description of the pathway awaits characterization of the *fem-2* gene product.

Alignment of the physical and genetic maps of *C. elegans* on the left end of Linkage Group III (Pilgrim, 1993) predicted the physical position of the *fem-2* gene. In this work, we describe how this has permitted transformation rescue of the *fem-2* mutant phenotype using clones selected from this genomic region. The DNA sequence of the rescuing region predicts a single open reading frame (ORF). It is likely that this sequence encodes *fem-2*, because mutant *fem-2* alleles show single basepair (bp) changes consistent with a defect in the predicted FEM-2 protein. This protein shows sequence similarity to several proteins involved in the regulation of signal transduction pathways. The two proteins most similar to FEM-2 are ABI1, a protein phosphatase from plants involved in mediating hormone response, and a protein of unknown function from humans. The sequence similarities suggest that FEM-2 may have protein phosphatase activity and that *C. elegans* sex determination may be regulated by a kinase/phosphatase cascade.

MATERIALS AND METHODS

Strains and Genetics

Nematode stocks were maintained and handled as described by Wood (1988). The strains used (Table 1) were maintained at 20°C, except where indicated. The isolation and characterization of some of the *fem-2* alleles used in this work are described by Kimble *et al.* (1984) and Hodgkin (1986). XO animals were isolated from strains carrying the *him-8* mutation, which produces a high incidence of males due to X-chromosome nondisjunction (Hodgkin and Brenner, 1977; Broverman and Meneely, 1994). Two putative deficiencies (Df) for the *fem-2* region have been generously shared in advance of

Table 1. *C. elegans* strains used in this work

Strain genotype	Source
fem-2(b245ts)	J. Hodgkin
fem-2(q117ts)	J. Kimble
fem-2(b245ts); him-8(e1489)	J. Hodgkin
fem-2(b245e2005); tra-3(e1767)	J. Hodgkin[a]
unc-45(r450ts) sDf124(s2670) + *sC1(s2073)[+ +dpy-1(s2171)]*	H. Stewart and D. Baillie
wcDf1(wc5)+ +dpy-1(e1)	L. Venolia
+ daf-7(e1372) par-2(e2030) + *unc-45(r450ts) sDf124(s2670) +; him-8(e1489)*	This laboratory
sC1(s2073)[+ +dpy-1(s2171)] him-8(e1489) *fem-2(e2105) + ; him-8(e1489)*	This laboratory[a]
sC1(s2073)[+ dpy-1(s2171)] him-8(e1489) *fem-2(e210n) +*	n = 1 to 6
sC1(s2073)[+ dpy-1(s2171)]	This laboratory[a]

[a] The isolation of b245e2005 and the e210n series of alleles are described in Hodgkin, 1986.

publication by H. Stewart and D. Baillie (*sDf124*) and L. Venolia [*wcDf1*].

XO animals hemizygous for *fem-2* mutant alleles were generated as follows: *fem*/+ males crossed to *fem*/*Df* females, or *Df*/+ males crossed to *fem*/*fem* females. No difference was seen in the phenotype of the XO animals prepared by these two methods. Homozygous *fem*/*fem* XO animals were produced among the self progeny of *fem-2(e2105); him-8(e1489)* maternally rescued hermaphrodites. The progeny were raised at 15, 20, or 25°C.

Nematode Transformation

Adult hermaphrodites, homozygous for *fem-2(b245ts)*, were raised at 20°C and were transformed with cloned DNA, using microinjection as described by Mello *et al.* (1991). The animals were injected with a mixture of the pRF4 plasmid, containing a dominant allele of the *rol-6* gene, *su1006dm* (Mello *et al.*, 1991) and the test DNA. The pRF4 plasmid causes the animals to adopt a dominant characteristic rolling motion on the plates, and serves as a positive control for transformation. Following injection, the hermaphrodites were incubated at the restrictive temperature of 25°C, unless indicated. Progeny from the injected animals were raised at 25°C, and examined 3 to 4 days later, for the Rol-6 phenotype, as well as for self-fertility. Uninjected *fem-2(b245ts)* animals, or animals injected with pRF4 alone produce an F1 brood at 25°C consisting exclusively of self-sterile females. In some experiments, the injected hermaphrodite was kept at 20°C, and lines that transmit the transgene were selected by following the Rol-6 phenotype alone. The transgenic lines were subsequently tested for rescue of the *fem-2(b245)* XX phenotype at 25°C. The number of transgenic animals scored varied from injection to injection and clone to clone, but at least two independent transmitting lines were assayed for each of the rescuing clones described in the text. For the clones that are classed as "failure to rescue," three to ten F1 Rol-6 animals were examined at the restrictive temperature. Only representative clones are discussed in the text. In addition to these, other clones have also been tested for their ability to rescue the *fem-2* mutant phenotype. The only clones that have shown rescue are those that include at least the minimal rescuing region.

Once the minimum rescuing region had been defined by the suppression of the *b245ts* XX germline defect, the DP#DBP026 and DP#DBP141 plasmids were tested for their ability to rescue the XX germline and XO germline and soma defects at 20 and 25°C. Heterozygous animals of the strain *fem-2(e2105) + /sC1[+ dpy-1(s2171)]; him-8(e1489)* were injected with the test and *rol-6* plasmids as described above. Transmitting lines were established at 20°C. Homozygous *fem-2(e2105); him-8* animals were selected from the progeny, and these were shown to produce both self-fertile XX animals and somatically normal males (XO) at 25°C. XO mating ability was scored by placing several Rol-6 XO animals (which had been raised at 25°C) on a plate with four *unc-45(r450ts)* hermaphrodites, and screening the F1 brood for non-Unc rolling males. (Several males were used because the extrachromosomal arrays are not completely meiotically or mitotically stable, and can produce mosaic progeny, and also because of the poor mating efficiency of *rol-6* males). For each of the plasmids, two independent matings produced outcross progeny, indicating that at least some of the *fem-2(e2105)* XO animals were completely rescued by the transgenes at 25°C.

DNA Analysis

DNA manipulation was as described in Pilgrim (1993) except where otherwise stated. DNA fragments were subcloned from cosmid and lambda genomic clones into pBluescript (Stratagene, La Jolla, CA). All clones were sequenced using a Sequenase v2.0 DNA sequencing kit (United States Biochemical, Cleveland, OH) as described by the manufacturer.

PCR Analysis

The presence of the test DNA in some, but not all, transgenic lines was assayed using DNA amplification by the polymerase chain reaction (PCR). Single worm PCR analysis was performed as described by Williams *et al.* (1992) and Pilgrim and Bell (1993). Confirmation of the 5′ end of the *fem-2* transcript used the rapid amplification of cDNA ends (RACE) technique (Frohman *et al.*, 1988) with a Life Technologies (Gaithersburg, MD) 5′ RACE kit, following the directions of the manufacturer. The primer DHA9 (Table 2) was used for first strand synthesis, and AMC1 was used for PCR amplification. The RACE product was cloned into the vector pGEM-T (Promega, Madison, WI) before sequencing.

The deletion of at least part of the *fem-2* locus by the deficiency *sDf124* was noticed by D. Collins and H. Stewart (personal communication) and confirmed in our lab. Heterozygous *sDf124*/+ hermaphrodites were placed on a plate and allowed to lay eggs for 24 h at 20°C. The adults were removed, and after a further 24 h, unhatched embryos were picked for PCR analysis. Embryos were picked from wild-type worms as a control. DNA from single embryos was prepared for PCR as described (Williams *et al.*, 1992). Primers DHA4, DHA17, DHA19, and SAD2 (Table 2) were used to test for DNA at the *fem-2* locus, while primers MMA1 and MMA2, which amplify DNA near the *unc-119* locus on the right end of Linkage group III (M. Maduro and D.P., unpublished data) were used as positive controls. A similar analysis was performed for the *wcDf1* deficiency.

DNA from the mutant alleles of *fem-2* was amplified as follows: for *b245ts* and *q117ts*, homozygous strains were grown at 20°C, and genomic DNA was isolated as described in Pilgrim (1993). For *e2103* and *e2105*, individual L4 hermaphrodites (either *fem*/+ or *fem*/*fem*, which are indistinguishable) from a *fem-2*/+ mother were set up on a plate at 20°C and allowed to produce an F1 brood. Six animals were selected from broods that consisted entirely of female adults (*fem-2*/*fem-2*), combined in one tube, and prepared as for the single worm PCR method described above. A portion of the DNA was amplified by PCR using the primers DHA3 and DHA4, and cloned into the pGEM-T (Promega) vector. For each mutant allele, clones from at least three independent PCR reactions were sequenced. For *e2101*, *e2102*, *e2104*, *e2106*, and *b245e2005*, genomic DNA was am-

Table 2. PCR primers used in this work

Primer	Sequence (5′ to 3′)	Position in fem-2 sequence[a]
AMC1	ACCTCGACGTCATAGCTAGT	464
DHA3	CAAAGATCTTGTCCCACCGAAGCCGGTAGTGG	278
DHA4	CGGTCTAGATGACGACGATATGGTGGATAG	2632
DHA9	CGTCTAGAAGCTCCAGCGAGCTTGCGAAGC	1524
DHA17	CCGAGCACTCGGAGATGTTC	1837
DHA19	CGATGGTCACGGTGGTCACGAG	1437
SAD2	AGAAATTCCATCACAAGCCAG	1912
MMA1	AGTCGGCCTTATTGTGCATTAC	n/a
MMA2	AAATTGCATGCCAGCACCGGTC	n/a

[a] Number refers to Figure 3, and most 3′ base in primer.

plified by PCR using the primers described above, and the PCR product was directly used as the template for sequencing.

cDNA Isolation and Analysis

A cDNA library, prepared from mixed stage *C. elegans* RNA and cloned into the λZap vector (Barstead and Waterston, 1989) was screened with a genomic DNA probe (15-kbp *Xba*I fragment containing sequences shown in Figure 2). From approximately 10^5 recombinant phage screened, 43 positive plaques were isolated. Subsequent characterization of the clones suggested that one-third came from within the 15-kb genomic region, but from outside the minimal rescuing region. Ten cDNA clones that fell entirely within the rescuing region were sequenced. One of the cDNA clones was chimeric, having been ligated to a second, unrelated cDNA. cDNA clones were excised in vivo to produce pBluescript SK(−) phagemid containing the cloned insert as described by the supplier (Stratagene).

Construction of Minigene

A partial minigene was constructed by ligating a 1.5-kb *Sal*I DNA fragment from a cDNA (containing part of exon 2 to the 3′ end of the cDNA, including the poly(A) tail and a portion of the polylinker) into a Bluescript plasmid containing 2.5-kb of upstream genomic sequence (*Xho*I–*Sal*I fragment) as well as exon 1, the first 56-bp intron, and part of exon 2. The cDNA fragment was cloned into both the sense and antisense orientations. The ability of the minigene to rescue the *fem-2(b245ts)* and *fem-2(e2105)* mutant phenotypes was tested as described above.

RNA Isolation and Characterization

Nematodes from N2 (XX) or *him-8(e1489)* (XX and XO) strains were grown in liquid cultures (mixed-stage or synchronized cultures) and purified according to Wood *et al.* (1988). Nematode pellets were then flash frozen in liquid nitrogen and stored at −80°C. RNA was isolated using glass bead homogenization as described by Hope (1994) and Johnson, Simpson, and Pilgrim (unpublished data). For embryos, RNA was prepared by grinding the frozen pellets with a mortar and pestle in liquid nitrogen. Northern analysis was performed using 1% agarose gels containing formaldehyde as described (Sambrook *et al.*, 1989). Radiolabelled antisense RNA probes were synthesized using T7 or T3 RNA polymerases as directed by the manufacturer (Promega), using a linearized cDNA clone as a template.

RESULTS

An alignment of the physical and genetic maps for the left end of *C. elegans* Linkage Group III (Pilgrim, 1993) suggested a molecular position for *fem-2*. The *fem-2* mutant alleles mapped between two RFLPs, *eP95* and *eP64*, which is a region of the physical map (approximately 0.2 map units or 50 kbp between the RFLPs; Pilgrim, 1993) that is completely covered by yeast artificial chromosome and cosmid clones from the *C. elegans* physical mapping project (Coulson *et al.*, 1986–

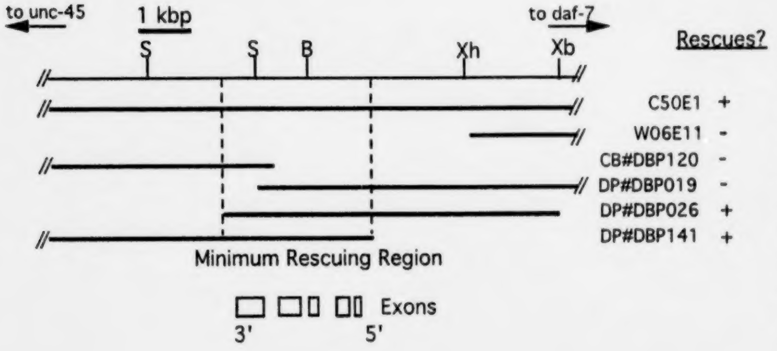

Figure 2. Restriction map of the *fem-2* region, aligned with the genetic map such that the left end of LGIII is to the left. Restriction enzyme recognition sites are shown above the line. Xb = *Xba*I; Xh = *Xho*I; B = *Bam*HI; S = *Sst*I restriction sites. The most informative subclones that have been tested for their ability to rescue the *fem-2* mutant phenotype are diagrammed, and indicated on the right. "+" indicates rescue, and "−" indicates failure to rescue by the criteria described (MATERIALS AND METHODS). The open boxes below the map indicate the positions of exons determined by sequencing the cDNA clones that mapped to the minimal rescuing region.

1991), as well as lambda clones identified by cross-hybridization to the yeast artificial chromosomes (Pilgrim, 1993). Cloned DNA fragments from this region of the physical map were tested for their ability to rescue the *fem-2* mutant phenotype, following germline transformation. The easiest *fem-2(b245ts)* mutant phenotype to score is the self-sterility of XX animals (females) at the restrictive temperature, due to their lack of spermatogenesis. Rescue was assayed by the restoration of self-fertility to homozygous *fem-2(b245ts)* transgenic animals at 25°C.

Three overlapping cosmid clones and one lambda clone were each able to restore spermatogenesis to transgenic animals raised at the nonpermissive temperature (Figure 2), while flanking cosmid and lambda clones failed to rescue. Transmitting lines were established with all of the rescuing clones, and several lines have been stably maintained for over 100 generations. The transgene is thought to be present as a meiotically unstable extrachromosomal array (Mello *et al.*, 1991) that is only passed to a fraction of the progeny. Animals that fail to inherit the array produce sterile progeny at the restrictive temperature. The rescuing region has subsequently been narrowed to 3.0 kbp, and its boundaries are defined by the ends of the subclones DP#DBP026 and DP#DBP141 (Figure 2). Clones containing less than this minimal region of genomic DNA fail to rescue. A subset of the rescuing clones have been successfully tested for XX germline rescue of a second temperature-sensitive allele *q117ts*, and a putative null allele *e2105*, both at 25°C.

The two minimal subclones DP#DBP026 and DP#DBP141 were then tested for their ability to rescue the *fem-2(e2105)* XO somatic and germline feminization at 25°C. At this temperature, *fem-2(e2105)* XO progeny of homozygous mothers develop as fertile females (Hodgkin, 1986). However, if they also carry either of the two plasmids as extrachromosomal arrays, the XO animals are somatically normal males, and are fertile when outcrossed (MATERIALS AND METHODS). Therefore, this confirms that the minimum rescuing region has been defined for both the somatic and germline phenotypes, in XX and XO animals, at all temperatures.

A 15-kbp genomic DNA fragment, which included the minimal rescuing region, was used to screen a mixed-stage cDNA library (Barstead and Waterston, 1989). Ten of the clones that mapped to the minimal rescuing region were characterized. Even though the small size and high A+T content of *C. elegans* introns (Wood *et al.*, 1988) allow the prediction of the position of exons within the genomic sequence; the intron/exon boundaries as well as the 5′ and 3′ ends of the gene were confirmed by sequencing the cDNAs. All clones were consistent with a single splicing pattern (see Figures 2 and 3), and were completely contained within the rescuing region. Both cDNA and genomic DNA sequences are given in Figure 3.

None of the cDNAs selected from the library were full length, although three extended into an apparent nematode spliced leader (SL1) sequence at the 5′ end and, therefore, contained all exons from the *fem-2* locus. The longest of the cDNA clones contained 9 bases at the 5′ end that matched the last bases of the SL1 sequence (Krause and Hirsh, 1987). The 5′ terminal segment was cloned using the PCR-based RACE procedure (Frohman *et al.*, 1988). Sequence analysis of the cloned RACE product confirmed that the SL1 leader is *trans*-spliced onto the *fem-2* mRNA. The 5′ end of the minimal rescuing region lies only 240 bp upstream of the spliced leader acceptor site. Because the transformation rescue assay for *fem-2* function produces multicopy transgenic arrays (Mello *et al.*, 1991), a basal level of transcription from each gene may prove sufficient to rescue, and therefore this small region may not contain all sequences responsible for wild-type *fem-2* transcriptional expression or regulation. The genomic and cDNA sequences are consistent with the presence of a single transcribed species, and a single transcript of the predicted size (1.8 kb) is seen on Northern blots (Figure 4). The developmental profile of the *fem-2* mRNA suggests that it is detectable at all stages of development, but is most abundant during adult development. When RNA prepared from cultures containing one-third XO animals is examined, no difference in the transcript patterns has been detected in embryos or adults (Figure 4 and our unpublished results). If there is a transcript from the *fem-2* locus that is specific to XO animals, it is either of low abundance, or co-migrates with the 1.8-kb transcript.

To further confirm that the cDNAs correspond to the *fem-2* locus, a minigene lacking all introns except the first was tested for its rescuing ability. The chimeric plasmids were constructed by fusing a genomic fragment containing 2.5 kbp of the upstream sequence, exon 1, intron 1, and part of exon 2 to a fragment from a cDNA clone containing the remainder of the exons and the poly(A) tail. In one chimera, the cDNA fragment was inserted in the "sense" orientation relative to the upstream region. In a second plasmid, the cDNA fragment was cloned in the "antisense" orientation. When transgenic lines were generated from the two chimeras, only the clone in the sense orientation was able to rescue the XX phenotype of *fem-2(b245ts)* at 25°C. The same sense clone is able to rescue the XO somatic and germline feminization of the *e2105* allele at 25°C, suggesting that no other transcript is necessary in XO animals. The second intron is almost completely composed of 12 repeats of a degenerate 30- to 35-bp sequence, itself an inverted repeat. Because this intron sequence can be removed with no apparent effect on the gene function, its role, if any, remains obscure.

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tttttgatccattttgatttgataaaaatgttattaatacaatacaatgcgaattaaattaatatttcgcgcgagaaacgcgcaacgcaccgaaagtgc   100
gcaaacgagaagctttgcgcgtttctggtgcagaaaattctgcattatttttttgttgatttttcctcatttgtgttgaaatccattgtttttctcgg   200

                                                                                                  Me
tttttctctgattttatgttaaaaacaattattgaaatgtcagATCAACCCAGCTGTCCCACCGAAGCCGGTAGTGGTTTTAAAACTGGAAAAATACAT   300

tGluLysValAsnGluGluArgAspAlaValPheGlu
                                                                                           AspHisIle
CGAAAAAGTAAACGAGGAGCGGGATGCGGTTTTCGAGgtagaaaacgggattttatatatttaatgctagttagaaaaatgtatattccagGATCACATC   400

GlyAspArgArgArgSerValArgSerLeuLeuGluGluAlaPheAlaAspGluMetGluLysThrSerTyrAspValGluValAlaAspThrProGlnP
GGCGATCGCCGTCGAAGTGTCCGGTCACTTCTGGAGGAGGCATTTGCAGATGAAATGGAGAAAACTAGCTATGACGTCGAGGTTGCCGACACTCCACAAC   500

roHisIleProIleArgPheArgHisProProIleAlaGlyProValHisAspValPheGlyAspAlaIleHisAspIlePheGlnLysMetMetLysAr
CGCATATTCCAATACGTTTCCGTCATCCACCAATCGCCGGACCAGTTCATGATGTTTTCGGAGACGCGATTCACGACATTTTCCAGAAAATGATGAAAAG   600

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AGGCCAGGCGGTCGACTTTTGTCACTGGGTGTCTCACTTGATCGCCACAGAAATTGACGAGAAATTCAGTGAAGTTGCGTTCAGAGATGTTCAGTATAAT   700

ProAspIleTyrValThrAspSerThrThrG
CCTGATATTTATGTTACCGATAGTACCACAGgtaattattaaatgtgcaaaatctgctattaatttagcagaccaagcatgggcctgctaaatattcagc   800
aggcctatccccattggtctgctaaatatttagcatacctaacttgggtctgctaaatatttagcagaccatccccattggtctgctaattatttagcag   900
accaactatgggtctgctaattatttagcagaccacctatgggtctgctaaaatatttagcatacctaccatgagtctgctaaatatttagcagacctat  1000
cctcattggtctgctaaatatttttagctggccattccccattgatctgctaattttttgcaaaaataaaacttgttaaatagaatttttaaaaaatcttc  1100

                                          luAlaLysLysLeuPheAsnAspLysIleTrpProAlaIleAspLysIleLeuGlnGl
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                                    q117 A        e2102 T
nAsnAlaGluThrCysProIleLeuSerGluLysTrpSerGlyIleHisValSerGlyAspGlnLeuLysGlyGlnArgHisLysGlnGluAspArgPhe
AAACGCCGAAACGTGCCCGATTCTGTCTGAAAAGTGGTCTGGAATCCACGTGTCGGGCGATCAACTGAAAGGGCAACGTCACAAGCAAGAAGATCGGTTT  1300

LeuAlaTyrProAsnGlyGlnTyrMetAspArgGlyGlu
                                                                                         AspProIleSe
TTGGCGTATCCTAATGGGCAATATATGGATCGTGGAGAGgtttaggaggattttaggcctggaaaattactgaaaaatgaaatttttagGATCCAATTTC  1400
                                                            e2005 A
rValLeuAlaValPheAspGlyHisGlyGlyHisGluCysSerGlnTyrAlaAlaGlyHisLeuTrpGluThrTrpLeuGluValArgLysSerArgAsp
GGTGTTAGCGGTGTTCGATGGTCACGGTGGTCACGAGTGCTCTCAGTACGCAGCCGGGCACCTTTGGGAGACATGGTTGGAGGTTCGAAAATCGCGAGAT  1500

ProSerAspSerLeuGluAspGlnLeuArgLysSerLeuGluLeuLeuAspGluArgMetThrValArgSerValLysGluCysTrpLysGlyGlySerT
CCTTCAGATAGCCTCGAAGATCAGCTTCGCAAGTCGCTGGAGCTTCTCGACGAACGAATGACTGTCAGAAGTGTGAAAGAGTGTTGGAAGGGTGGAAGTA  1600

hrAlaValCysCysAlaIleAspMetAspGlnLysLeuMetAlaLeuAlaTrpLeuGlyAspSerProGlyTyrValMetSerAsnIleGluPheArgGl
CAGCTGTCTGCTGTGCAATCGACATGGATCAGAAGCTTATGGCGCTGGCGTGGCTCGGTGACTCACCCGGATACGTCATGTCGAACATTGAATTCCGTCA  1700

nLeuThrArgGlyHisSerProSerAspGluArgGluAlaArgArgValGluGluAlaGlyGlyGlnLeuPheValIleGlyGlyGluLeuArgValAsn
GTTGACACGTGGACACTCGCCATCCGATGAACGGGAAGCTCGGCGTGTTGAGGAAGCTGGCGGCCAGCTTTTTGTGATTGGAGGCGAATTGCGGGTGAAC  1800

                      b245 A
GlyValLeuAsnLeuThrArgAlaLeuGlyAspValProGlyArgProMetIleSerAsnGluProGluThrCysGlnValProIleGluSerSerAspT
GGAGTTCTAAATCTCACCCGAGCACTCGGAGATGTTCCTGGACGGCCGATGATCTCTAATGAACCGGAAACGTGTCAGGTGCCTATTGAAAGCTCGGATT  1900

yrLeuValLeuLeuAlaCysAspGlyIleSerAspValPheAsnGluArgASpLeuTyrGlnLeuValGluAlaPheAlaAsnAspTyrProValGluA
ATCTGGTGCTCCTGGCTTGTGATGGAATTTCTGATGTTTTCAACGAGCGTGACCTGTACCAGTTGGTGGAGGCATTTGCGAATGATTATCCTGTTGAAGg  2000
ttaataaaaattcttggagaaaaactaaatttccaaagccaaaaactatttttcgtttttttttctgaaaaaaatcagaagattggctatttctcggg  2100
ggttttcaaaaaaaaaaagtaaagattttcgaattattgtatttttttaaaaaagaaaatcgtgaatttactaaaatttgaaaaaatcaatttttaaaaaa  2200

                                                                                                  sp
accattcaaaattatttttaaggtaaaaaacattagccgacgttactttatttttttaatgtagaaaattctttaaaaatcgaaaaaaaatttttcagAC  2300

TyrAlaGluLeuSerArgPheIleCysThrLysAlaIleGluAlaGlySerAlaAspAsnValSerValValIleGlyPheLeuArgProProGlnAspV
TACGCGGAGCTCTCCCGTTTCATCTGTACAAAAGCGATTGAGGCTGGAAGTGCCGACAACGTGTCTGTTGTGATTGGCTTTTTGCGTCCACCGCAGGATG  2400

alTrpLysLeuMetLysHisGluSerAspAspGluAspSerAspValThrAspGluGlu***
TGTGGAAGCTTATGAAACATGAATCAGACGATGAAGATTCCGATGTCACTGATGAGGAATAACTGCTTTTCGGTGGAAATTTTGCCTGAAAATTGGGAAA  2500
ATTCTTGCTAAACTTCGGTTGATTTTCTCAAATTTTTGTCGTTTTTTTTCCTAATTTTCGGTCGATTTTATCAAATTTTCCCCTGTTTTCCTGTGTCTAT  2600
TTGTTTTTTGGGTATTCCAATTTTCTCTCTCCTATTCACCATATCGTCGTCATTTCTCCCCGTTATCTAGCAGCTTTCCGTGACCCACACACATATTTTT  2700
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CATATATTTGATTTGAATTTATTTTATGATATAAATTGTATAATGACGTGGAAttactgggatattgagtagctgaataggttttgagacacttttgaca  2900
ctgatttcataatgactgtaactttcaactcgtgaaaatgtaaaaatcggagtataccagtcattttcagtagaaaatctccttttcaattgaaaatccg  3000
ggatttttcgaaagaaaagctcgaaaacaccgcgaaaaaatgattaaaaaagtcatattttcaagagggaatttcaaatttcccgcttaca
```

Figure 3. DNA sequence of the minimal rescuing region of the *fem-2* gene. Exons are shown in capital letters, introns and flanking regions are shown in lower case. Numbers refer to nucleotides of genomic DNA from the beginning of the rescuing region. Predicted translation is given above the sequence, and the predicted initiation codon is underlined. The DNA changes in the *b245*, *q117*, *e2102*, and predicted *e2005* mutant alleles are indicated in boldface above the protein sequence. The *e2005* mutation is inferred from the sequencing of the doubly mutant *b245e2005* allele as described in the text. GenBank accession number for this sequence is U29515.

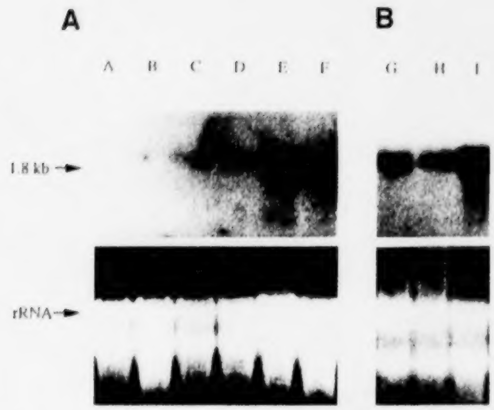

Figure 4. Northern blot of total *C. elegans* RNA. Fifteen micrograms of total RNA, prepared from wild-type hermaphrodite (lanes A-G and I) or *him-8(e1489)* XX and XO (lane H) worms synchronized at each developmental stage (embryo, first to fourth larval [L] stages, and adult), was loaded in each lane. Following electrophoresis and capillary transfer, the blot was hybridized to an antisense riboprobe prepared from a *fem-2* cDNA clone. The top panel shows the resulting autoradiogram. The bottom panel shows the ethidium bromide-stained gel before Northern transfer to show relative loading. Lanes A, G, and H are embryo RNA. Lane B is L1, C is L2, D is L3, E is L4, and F and I are adult RNA. The blot in panel B was hybridized with a probe of higher specific activity than the blot in panel A to allow more sensitive detection of the embryonic mRNA.

The sequence of the cDNAs representing the *fem-2* transcript is shown in Figure 3. Assuming that the first in-frame ATG sequence corresponds to the initiator methionine, the single open reading frame encodes a protein of 449 amino acids. In a BLAST search of the protein sequence databases (Altschul *et al.*, 1990), six separate regions of the potential FEM-2 protein (conserved motifs I-VI in Figure 5) show significant similarity to an uncharacterized ORF from humans, as well as several mammalian and yeast protein phosphatase 2C (PP2C) genes (Figure 5). In addition, the ABI1 gene from the plant *Arabidopsis thaliana*, which is involved in regulation of the plant cell's response to abscisic acid (Leung *et al.*, 1994; Meyer *et al.*, 1994), also shows similarity over the same regions. A second *Arabidopsis* phosphatase, KAPP (Stone *et al.*, 1994), also shows similarity to FEM-2, but at a lower level. When comparing the predicted amino acid sequence of FEM-2 with that of the other proteins over the six motifs, the human ORF is 37% identical and 56% similar over 190 amino acids, rat PP2C is 37% identical and 49% similar over 181 amino acids, and ABI1 is 31% identical and 46% similar over 189 amino acids.

The amino terminal third of the predicted FEM-2 protein showed no significant similarity to any sequence in the database, as judged by the BLAST program. Most of the "classical" PP2C proteins have short (30–40 amino acids) amino terminal regions proximal to motif I. In contrast, FEM-2, ABI1, and the human ORF have much larger amino terminal regions, extending 135–170 residues amino terminal to motif I (Figure 5). The amino terminal domains of the three proteins have no significant sequence alignment with one another, although all are rich in charged amino acids and proline. FEM-2 also shows protein sequence similarity to another predicted *C. elegans* protein, F42G9.1, which corresponds to the first ORF from cosmid clone F42G9 sequenced by the *C. elegans* genome sequencing project (Sulston *et al.*, 1992; Wilson *et al.*, 1994). Cosmid F42G9 has been placed on the physical map at the left end of Linkage Group III, close to *fem-2* on the *eP64* contig, about 100 kbp toward *daf-7* (Pilgrim, 1993). (The F42G9 cosmid is colinear with cosmid F10H6 in Figure 5 of Pilgrim, 1993). The sequence identity between FEM-2 and F42G9.1 is restricted to the conserved motifs, suggesting that the two proteins are distantly related (Figure 5). The predicted F42G9.1 product appears to contain a large in-frame insertion between motifs II and III, relative to all the other homologues. Because the transcript from this region has not been examined, it is possible that this represents a cryptic intron.

Figure 5 shows a cartoon of the alignment of the protein homologues. The proteins fall into three structural classes, based on the following criteria: 1) the presence of an extended amino-terminal domain (Class A); 2) the presence of an "Asp-Trp" dipeptide at the end of motif I (Class B and C); and 3) the presence of an extended carboxy terminus and a conserved acidic 9- or 10-amino acid peptide between motifs IV and V (Class C: ILRAEEDEF in the rat PP2C β isoform, IERSEEDDQF in the human α isoform, and EALTPEDEF in F42G9.1).

Twelve *fem-2* mutant alleles have been isolated and genetically characterized (Kimble *et al.*, 1984; Hodgkin, 1986). Two of the existing alleles are temperature sensitive, and at least four others are thought to have two mutations in the *fem-2* gene (Hodgkin, 1986). All of the putative *e2101* to *e2106* single mutants were isolated *in trans* to a doubly mutated allele *b245e2005* (Hodgkin, 1986). Of these alleles, *e2105* has been proposed as a candidate for a null allele (Hodgkin, 1986). However, because homozygous *fem-2(e2105)* XO animals are completely feminized only at 25°C, the possibility remains that the *e2105* allele retains some low level of Fem-2 activity. The recent isolation of genetic deficiencies for portions of the left end of Linkage Group III (H. Stewart and D. Baillie, personal communication; L. Venolia, personal communication) allowed a more definitive test for a null allele. One of the predicted characteristics of a null allele is that the phenotype of the allele *in trans* to a deficiency for the locus is no more severe than the phenotype of the homozygous

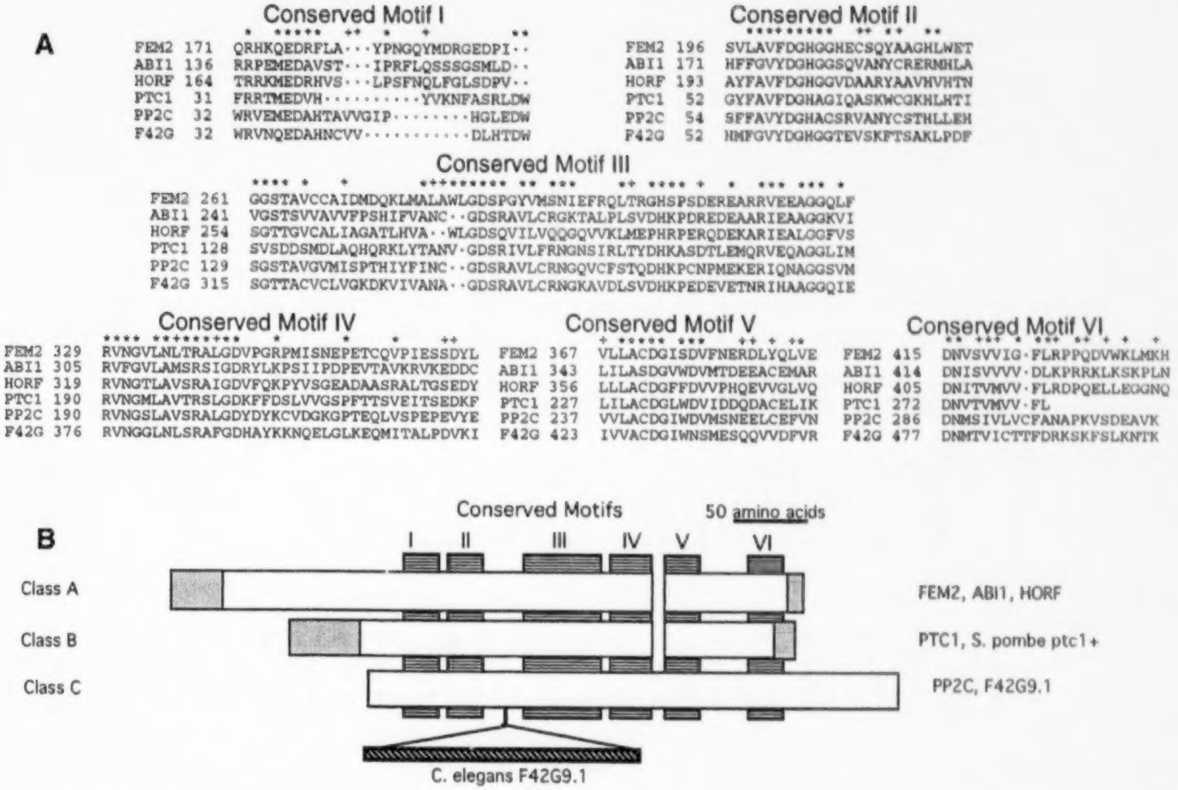
A
Conserved Motif I
FEM2 171 QRHKQEDRFLA···YPNGQYMDRGEDPI··
ABI1 136 RRPEMEDAVST···IPRFLQSSSGSMLD··
HORF 164 TRRKMEDRHVS···LPSFNQLFGLSDPV··
PTC1 31 FRRTMEDVH··········YVKNFASRLDW
PP2C 32 WRVEMEDAHTAVVGIP·······HGLEDW
F42G 32 WRVNQEDAHNCVV··········DLHTDW
Conserved Motif II
FEM2 196 SVLAVFDGHGGHECSQYAAGHLWET
ABI1 171 HFFGVYDGHGGSQVANYCRERNHLA
HORF 193 AYFAVFDGHGGVDAARYAAVHVHTN
PTC1 52 GYFAVFDGHAGIQASKWCGKHLHTI
PP2C 54 SFFAVYDGHACSRVANYCSTHLLEH
F42G 52 HMFGVYDGHGGTEVSKFTSAKLPDF
Conserved Motif III
FEM2 261 GGSTAVCCAIDMDQKLMALAWLGDSPGYVMSNIEFRQLTRGHSPSDEREARRVEEAGGQLF
ABI1 241 VGSTSVVAVVFPSHIFVANC··GDSRAVLCRGKTALPLSVDHKPDREDEAARIEAAGGKVI
HORF 254 SGTTGVCALIAGATLHVA··WLGDSQVILVQQGQVVKLMEPHRPERQDEKARIEALGGFVS
PTC1 128 SVSDDSMDLAQHQRKLYTANV·GDSRIVLFRNGNSIRLTYDHKASDTLEMQRVEQAGGLIM
PP2C 129 SGSTAVGVMISPTHIYFINC··GDSRAVLCRNGQVCFSTQDHKPCNPMEKERIQNAGGSVM
F42G 315 SGTTACVCLVGKDKVIVANA··GDSRAVLCRNGKAVDLSVDHKPEDEVETNRIHAAGGQIE
Conserved Motif IV
FEM2 329 RVNGVLNLTRALGDVPGRPMISNEPETCQVPIESSDYL
ABI1 305 RVFGVLAMSRSIGDRYLKPSIIPDPEVTAVKRVKEDDC
HORF 319 RVNGTLAVSRAIGDVFQKPYVSGEADAASRALTGSEDY
PTC1 190 RVNGMLAVTRSLGDKFFDSLVVGSPFTTSVEITSEDKF
PP2C 190 RVNGSLAVSRALGDYDYKCVDGKGPTEQLVSPEPEVYE
F42G 376 RVNGGLNLSRAFGDHAYKKNQELGLKEQMITALPDVKI
Conserved Motif V
FEM2 367 VLLACDGISDVFNERDLYQLVE
ABI1 343 LILASDGVWDVMTDEEACEMAR
HORF 356 LLLACDGFFDVVPHQEVVGLVQ
PTC1 227 LILACDGLWDVIDDQDACELIK
PP2C 237 VVLACDGIWDVMSNEELCEFVN
F42G 423 IVVACDGIWNSMESQQVVDFVR
Conserved Motif VI
FEM2 415 DNVSVVIG·FLRPPQDVWKLMKH
ABI1 414 DNISVVVV·DLKPRRKLKSKPLN
HORF 405 DNITVMVV·FLRDPQELLEGGNQ
PTC1 272 DNVTVMVV·FL
PP2C 286 DNMSIVLVCFANAPKVSDEAVK
F42G 477 DNMTVICTTFDRKSKFSLKNTK
B
Conserved Motifs
50 amino acids
I
II
III
IV
V
VI
Class A
Class B
Class C
FEM2, ABI1, HORF
PTC1, S. pombe ptc1+
PP2C, F42G9.1
C. elegans F42G9.1

Figure 5. FEM-2 alignment with putative homologues. (A) Alignment of putative homologues to illustrate conserved motifs. Sequences are as follows: FEM2, predicted protein from *fem-2* locus; ABI1, *ABI1* protein from *Arabidopsis thaliana* (Leung *et al.*, 1994; Meyer *et al.*, 1994); HORF, predicted human protein from cDNA sequence (Nomura *et al.*, 1993); PTC1, *Saccharomyces cerevisiae* product of *PTC1* gene (Maeda *et al.*, 1993); PP2C, rat protein phosphatase 2C β isoform (Wenk *et al.*, 1992); F42G, F42G9.1 ORF from *C. elegans* sequencing project (Wilson *et al.*, 1994). * = >60% of proteins have same amino acid; + = >80% of proteins have one of two amino acids; • = gap introduced to maximize alignment. Numbers refer to the residue number at the beginning of the motif. Other mammalian PP2C homologues are similar to the P2CB sequence. Motifs IV and V are contiguous in FEM-2, *ABI1*, HORF, and *PTC1*, but are separated by a 9- to 10-amino acid peptide in the mammalian PP2C homologues and F42G9.1 (ILRAEEDEF in the rat PP2C β isoform, IERSEEDDQF in the human α isoform, and EALTPEDEF in F42G9.1). Sequence accession numbers: *ABI1*, gp X77116; HORF, gp D13640; *PTC1*, sp P35182; PP2C, sp P35815; and F42G9.1, gp U00051. (B) Alignment of proteins to show relative positions of conserved motifs I-VI. Stippled boxes represent position and extent of the conserved motifs. Open boxes represent protein sequences, shaded boxes represent extensions that are not present in all members of a class. The dark hashed box is the predicted extra sequence in the *C. elegans* F42G9.1 protein that lies between motifs II and III. Motif IV and V are colinear in Class A and B proteins, but separated by a 9- to 10-amino acid peptide in Class C proteins. The spacing between motif V and VI varies between the proteins.

null allele. Embryos homozygous for either the *sDf124* or *wcDf1* deficiencies fail to show a PCR product with two different primers sets from the *fem-2* coding region (Figure 6), although primers corresponding to sequences elsewhere in the genome are able to amplify products Therefore, both deficiencies appear to delete much if not all of the *fem-2* gene.

The *e2105* allele was placed *in trans* with a deficiency, and the phenotype of the resulting animals was examined. In the XO, the requirement for Fem-2 activity is least stringent at lower temperatures (Hodgkin, 1986). At 20°C, many *e2105* XO homozygotes die during development (the animals most often break open due to a weakening in the ventral hypodermis), but a few survive, and they have abnormal gonads, containing oocyte-like cells. These animals also have rudiments of the male tail, with a small fan. Rays are often present (Figure 7; Hodgkin, 1986) and the animals are sterile. At 15°C, the feminization of XO animals is weaker (Figure 7). The gonad has a typically female shape, but only rarely are oocyte-like cells seen. These animals also have projections or bulges in the ventral midbody region, where the vulva would

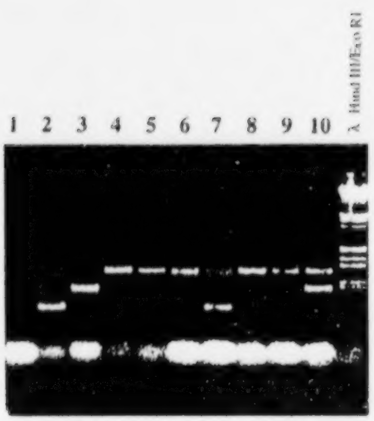

Figure 6. PCR analysis of putative deficiencies for the *fem-2* region. DNA was prepared from embryos that were wild type for *fem-2* (lanes 1–4, 7, and 10), homozygous for *wcDf1* (lanes 5 and 8), or homozygous for *sDf124* (lanes 6 and 9). Three primer sets were used to PCR amplify the DNA. Two sets correspond to *fem-2* (SAD2-DHA19 and DHA4-DHA17) whereas the third set amplifies a region of the unlinked *unc-119* gene (MMA1-MMA2). Lane 1 contains one primer from each of the three primer sets (DHA4, MMA1, and SAD2). Lanes 2–4 show the bands amplified for each of the three primer sets alone on wild-type DNA (SAD2-DHA19, DHA4-DHA17, and MMA1-MMA2, respectively). Lanes 5–7 show amplified products of two primer sets (SAD2-DHA19 and MMA1-MMA2) with *wcDf1*, wild-type, and *sDf124* embryos. Lanes 8–10 show amplified products of the primer sets DHA4-DHA17 and MMA1-MMA2, again with *wcDf1*, wild-type, and *sDf124* embryos. Each lane represents the PCR results from a single embryo.

normally form in a female. The fan and rays in the tail are more wild type. Although the XO phenotype can vary from animal to animal, the range of feminization seen in hemizygous *e2105/Df* XO animals is indistinguishable from *e2105* homozygotes at either temperature (Figure 7). Identical results were seen with *e2105 in trans* to either *sDf124* or *wcDf1*. Because one of the tests for a null allele is that the homozygous and hemizygous phenotypes are identical, this suggests that *e2105* is a putative null mutation for the *fem-2* gene.

The DNA encoding the *fem-2* locus (between nucleotide positions 250 and 2650; Figure 3) was sequenced from eight of the mutant *fem-2* alleles (*b245ts*, *q117ts*, and *e2101–6*), and from two different strains containing wild-type *fem-2* genes. All alleles were reportedly induced using ethylmethane sulfonate (EMS), which causes primarily transition mutations of G-C to A-T bp. Both PCR amplified wild-type alleles showed an identical sequence to that determined from the clones isolated from the genomic and cDNA libraries. All mutant alleles showed base changes when compared with the wild-type sequence. The positions of the mutations in these alleles are shown in Figure 3. The *q117ts* allele contains a single G-C to A-T base change at position 1242, which is predicted to cause the substitution of glutamic acid for glycine. The *b245ts* allele contains a single G-C to A-T change at position 1828, predicted to cause a substitution of arginine for glycine at amino acid 341 in motif IV. This position is absolutely conserved among all homologues shown. The *e2102* allele contains a single G-C to A-T change at position 1262, creating an in-frame stop codon.

The *e2101*, *e2103*, *e2104*, *e2105*, and *e2106* sequences were identical to one another, and unexpectedly showed two base changes from wild type. One change was identical to the *b245* mutation at position 1828. The other was a G-C to A-T transition at position 1466, which creates an in-frame stop codon (Figure 3). These alleles (along with *e2102*) were isolated in a non-complementation screen in a strain containing the *b245e2005* double mutation (Hodgkin, 1986). Although the entire DNA sequence from this allele has not been determined, *b245e2005* also shows the *b245*' change at position 1828, and the "stop" at position 1466. It therefore appears that the *e2101*, *-3*, *-4*, *-5*, and *-6* alleles were not "new" *fem-2* alleles, but recombinational reisolates of *b245e2005*. Regardless of their origin, both the presence of a translational stop codon, and the hemizygous phenotype support the contention that these represent the null phenotype of *fem-2*.

DISCUSSION

The *fem-2* gene product acts in the sex determination hierarchy, as a positive regulator of the male fate (spermatogenesis) in the germ line, and as a negative regulator of the female fate in the soma. If all genes downstream of *tra-2* in the epistatic pathway act in a cell autonomous manner, the interactions in the soma can be modeled as a signal transduction pathway, culminating in the TRA-1 transcriptional regulator protein (Figure 1). The genetic epistasis suggests the following: 1) that the *tra-2* and *tra-3* genes negatively regulate the *fem* genes, and 2) that the *fem* genes negatively regulate the activity of *tra-1*. The activity of all three of the *fem* genes seems to be required for normal male development, but it is not known how, or if, they interact. FEM-2 may play different regulatory roles in the germ line and in the soma. Loss-of-function (lf) mutations in *tra-1* suppress the somatic feminization caused by *fem-2* mutations (Hodgkin, 1986), but Fem-2 activity is still required for spermatogenesis at 25°C (Hodgkin, 1986). *fem-3* gain-of-function (gf) mutations suppress the germline feminization caused by a temperature-sensitive *fem-2* mutation, but do not affect the soma (Barton *et al.*, 1987).

The *fem-2* locus has been cloned based upon two lines of evidence. First, transgenes that contain the intact coding region for the putative FEM-2 protein are necessary and sufficient to rescue the mutant phenotype in both germline and somatic tissues, in both XX

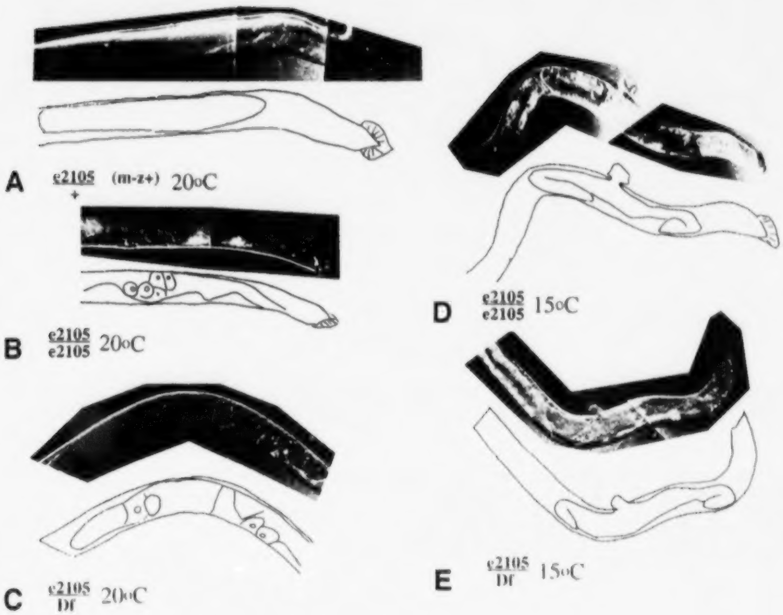

Figure 7. Photographs using differential interference contrast microscopy (top) and cartoons of the photographs (bottom) of the *fem-2(e2105)* heterozygous, homozygous, and hemizygous XO phenotypes at different temperatures. All animals were progeny from *fem/fem* mothers. In panels B and C, there are oocyte-like cells apparent in the gonad. The deficiency used in these examples was *sDf124*, but identical results were seen when *wcDf1* was used instead. The XO phenotype can be quite variable, so the examples shown do not portray all the defects seen in animals of the same genotype. Although the tail phenotype is not very clear from these examples, there were no apparent differences in the ranges of defects seen in the homozygous vs. hemizygous animals.

and XO animals. The minimal rescuing region includes 300 bp 5′ to the predicted translation start codon, and 600 bp 3′ to the predicted translation termination codon. Second, all mutant alleles of *fem-2* that were examined contain base changes within the predicted coding region, compared with two different wild-type alleles as well as to cDNA and genomic DNA clones from the physical mapping project. The base changes are predicted to result in alterations of amino acid sequence in all cases, and are consistent with the mutations resulting from EMS mutagenesis. Two different chromosomal deletions that remove at least part of the predicted *fem-2* coding region fail to complement the *fem-2(e2105)* allele.

The transgenic rescue results with genomic and cDNA clones suggest that a single transcription unit is sufficient to account for all the functions of *fem-2* in XX and XO animals. There is a single transcript from the locus in XX animals, which is detected throughout development, but is most abundant in adults. Although this may seem unusual given the masculinizing role of *fem-2*, and the observation that Fem-2 activity is completely dispensable for adult female development, the *fem-2* mutant phenotype can be maternally rescued (only *fem-2/fem-2* progeny of *fem-2/fem-2* mothers are completely feminized), suggesting that the FEM-2 protein or its mRNA can be inherited through the female germ line. The high expression of *fem-2* mRNA in the adult hermaphrodite may represent transcription that is restricted to the germ line. Somatic expression of *fem-2* is predicted in males throughout their life (Kimble *et al.*, 1984). In mixed male/hermaphrodite cultures, no other transcript has yet been detected.

The sequence similarity found in this work between the predicted FEM-2 protein and protein phosphatase 2C (PP2C) homologues suggests that FEM-2 might have phosphatase activity. PP2C enzymes have been characterized as serine/threonine specific phosphatases that are Mg^{+2} dependent, and resistant to okadaic acid (Cohen, 1989), but their regulatory role is poorly understood. Maeda *et al.* (1993 and 1994) showed that the yeast homologue *PTC1* is an essential gene in yeast under certain conditions, and suggested that *PTC1* is involved in the regulation of a two-component signal transduction system, possibly via a mitogen-activated protein kinase homologue involved in osmosensing. *S. cerevisiae* has at least three separate mitogen-activated protein kinase cascades (reviewed in Nieman, 1993). The mutant phenotypes of genes involved in each separate yeast pathway do not show obvious pleiotropic defects, suggesting that there is little or no interaction between the pathways under normal circumstances. None of the other proteins in these pathways show similarity to any of the gene products in the *C. elegans* sex determination pathway.

Another apparent homologue of FEM-2 is the *ABI1* gene of *Arabidopsis thaliana*. The *abi1* mutant is insensitive to the phytohormone abscisic acid (ABA) (Koornneef *et al.*, 1984). ABA is involved in many aspects of plant growth and development. In at least one fern species, ABA-resistant mutations affect sex determination (Banks, 1994). The *abi1* mutant phenotype in *Arabidopsis* has been proposed to result from a lack of negative feedback regulation in an intracellular step of hormone signaling (Leung *et al.*, 1994; Meyer *et al.*, 1994). The mutation in the *abi1–1* allele affects a conserved glycine in motif II, suggesting a role for this motif in the negative regulation of the phosphatase activity (Leung *et al.*, 1994; Meyer *et al.*, 1994). *ABI1* has been shown to have protein phosphatase function in vitro (Leung *et al.*, 1994; Meyer *et al.*, 1994). Again, although FEM-2, *PTC1*, and *ABI1* appear to have roles in the regulation of signal transduction pathways, there is no evidence that any other components in the signaling pathways are common to the systems.

Despite the similarity between FEM-2 and canonical mammalian PP2C enzymes, FEM-2 is most similar in sequence to an unidentified human ORF. With the long amino-terminal domain, short carboxy-terminal domain, and lack of a conserved decapeptide between domains IV and V, FEM-2 (along with *ABI1* and the human ORF) appears to fall into a distinct class of PP2C homologues. The presence of the long amino terminal domain suggests that the three proteins may regulate their protein activity in a manner different from the classical PP2C proteins. The Arabidopsis KAPP phosphatase also appears to be a Class A PP2C enzyme, based upon its long amino-terminal domain (320 amino acids; Stone *et al.*, 1994). In this case the amino-terminal domain appears to directly interact with the receptor serine/threonine kinase RLK5. The F42G9.1 protein from *C. elegans* is more similar in structure to the canonical mammalian PP2C proteins. F42G9.1 does not have the long amino terminus found in FEM-2 and ABI1, but does have the decapeptide between domains IV and V, which makes it most similar to the mammalian PP2C isoforms. No genetic loci have yet been identified that map to the predicted position of F42G9.1, and the existence of a protein product has not been confirmed.

Could the sex determination pathway be regulated by phosphorylation? There are several well studied paradigms for regulation of transcription by phosphorylation (Hunter and Karin, 1992). For example, the nuclear localization of the transcription factor NF-[κ]B is controlled by the phosphorylation of its regulatory subunit I[κ]B (Ghosh and Baltimore, 1990). I[κ]B contains ankyrin repeats (Haskill *et al.*, 1991), like FEM-1, therefore the localization of TRA-1 may be controlled by interactions with FEM-1 to control somatic sex. FEM-2 may regulate such an interaction by dephosphorylating FEM-1. However, there is no evidence to suggest that FEM-1 and TRA-1 interact, and limited evidence that TRA-1 is always nuclearly localized (D. Zarkower, personal communication). An alternative model is that *fem-3* product may be the target. The predicted FEM-3 protein contains several putative casein kinase phosphorylation sites (Ahringer *et al.*, 1992) but it is not known whether any of these are used in vivo or in vitro. In addition, *fem-3* gain-of-function mutations have been shown to suppress the *fem-2* phenotype of the conditional allele *b245* in the germ line, but not the soma (Barton *et al.*, 1987). These gain-of-function mutations alter a control sequence in the *fem-3* 3'-untranslated region (UTR) of the mRNA (Ahringer and Kimble, 1991; Evans *et al.*, 1992). Perhaps FEM-2 is a molecule that normally controls *fem-3* translation in the germ line, possibly by de-phosphorylating an unidentified untranslated region binding factor. The suppression has only been seen for one allele of *fem-2*, and is limited to the germ line and therefore, this model does not explain all the observations. A third possibility is that the TRA-1 protein, which has several putative phosphorylation sites, is the target (Zarkower and Hodgkin, 1992). In this model, there must be a second target in the germ cells, where *fem-2* activity is normally required and is independent of *tra-1*. Because *fem-2* has different genetic roles in the germ line and soma, neither of these speculations can be ruled out.

A further complication to these models is the absence of a characterized kinase in the sex determination pathway. Because *fem-2* mutants show defects in sex determination in both the germ line and soma, one might expect that an associated kinase must exist in the two tissues. If the activity of FEM-2 is to remove phosphate groups from a protein target to produce the "male" phenotype, a protein kinase must initially phosphorylate the target. No kinase has been found among the genes of the pathway; if an associated kinase exists, loss-of-function mutations in the gene are either rare, or do not lead to a simple sexual transformation phenotype (the predicted phenotype of such a mutation would be "Tra" phenotype of masculinization). Perhaps the kinase is constitutive, and essential, or redundant. Alternatively, one of the characterized genes in the pathway may have a kinase activity that cannot be predicted from the primary sequence.

Finally, it is clear that under some circumstances, the FEM-2 product is dispensable for normal male development. There is evidence from the current work that the strongest alleles of *fem-2* completely eliminate FEM-2 activity. The sequence of the *e2102* and *e2105* alleles predicts a truncated protein, missing much of the PP2C conserved region. The phenotype of the *e2105* allele gets no stronger when *in trans* to a deficiency, suggesting that there is no partial function supplied by one copy of *e2105*. Yet in *fem-2(e2105)* animals, male somatic structures as well as sperm can still be formed in certain conditions (this work; Hodgkin, 1986). Therefore, *C. elegans* can bypass the requirement for *fem-2* activity. If FEM-2 is a phosphatase whose activity leads to the activation of spermatogenesis and the inactivation of TRA-1 in the soma, such dephosphorylation is not an essential part of the regulation. Identification of the cellular targets of FEM-2 activity will help to clarify its apparent dual role in somatic and germline sex determination.

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